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# Temperature-dependent sex determination and the evolutionary potential for sex ratio in the painted turtle, Chrysemys picta

Carrie Lynne Morjan
*Iowa State University*

# Temperature-dependent sex determination and the evolutionary potential for sex ratio in the painted turtle, *Chrysemys picta*

by

**Carrie Lynne Morjan**

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Program of Study Committee:
Fredric J. Janzen, Major Professor
William R. Clark
Gavin J. P. Naylor
Edward Pollak
Carol M. Vleck

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Carrie Lynne Morjan

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

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For the Major Program

I dedicate this work to:

My husband and best friend, Wilmar

My mom, Ann

and in loving memory of Austin K. Bridges

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## ABSTRACT

Most vertebrates exhibit genotypic sex determination (GSD), where sex ratios are determined by the segregation ratio of sex chromosomes. However, many reptiles exhibit temperature-dependent sex determination (TSD), a sex-determining mechanism in which the incubation environment permanently determines sex of developing offspring. Traditionally, skewed sex ratios are generally considered maladaptive, and GSD is thought to have an adaptive advantage over TSD. This research had two main objectives: (1) to evaluate the roles of two traits (nesting behavior by females and offspring sex ratios in response to thermal incubation conditions) thought to be important for the maintenance of sex ratios in this system, using the painted turtle (*Chrysemys picta*), and (2) evaluate the adaptive significance of TSD in a genotypic sex determining system.

Observations on the nesting behavior of painted turtles in a natural population in Illinois demonstrated that females did not nest in environments exhibiting extreme soil surface temperatures. Neither was a key behavior used during nesting, "ground-nuzzling," associated with soil surface temperatures. These results suggest that either females in this population do not use soil surface temperature as a cue for selecting nest sites, or select sites with intermediate soil surface temperatures that may be less likely to bias sex ratios.

Geographic comparisons from two populations of *C. picta* inhabiting differing climates (Illinois and New Mexico) demonstrated that the New Mexico population experiencing a hotter climate exhibited a significantly higher pivotal temperature (temperature producing a 1:1 sex ratio) than the Illinois population for laboratory-incubated eggs. However, this difference was small compared to differences in climatic conditions experienced by the population. Furthermore, 1:1 sex ratios were not produced when eggs

were incubated at these calculated pivotal temperatures during a subsequent nesting season, possibly due to seasonal effects on sex determination for both populations.

Female *C. picta* from Illinois and New Mexico nested at similar times during two field seasons, and nests from both populations experienced similar nest temperatures despite a relatively hot year in New Mexico. Nests in New Mexico were laid under little or no vegetation cover (a microhabitat variable that directly reduced nest temperatures), but were placed at sites likely to experience high soil moisture, which also reduced nest temperatures.

In simulation models incorporating realistic levels of climatic and phenotypic variation taken from a natural population of *C. picta*, pivotal temperatures evolved more rapidly than did nest-site choice by females in response to perturbed sex ratios. Females had little ability to influence offspring sex ratios because of the relatively strong effects of yearly climate on nest temperatures. Natal philopatry to nest sites also caused maladaptive nesting behavior in terms of Fisherian sex ratio selection.

Simulation models demonstrated that TSD can invade a population exhibiting GSD and reach fixation through several avenues that do not include a widely accepted adaptive function for TSD (the Charnov-Bull model). For example, TSD may evolve as a relatively neutral mechanism with no adaptive significance in long-lived organisms, or by interfering with the original GSD mechanism.

Results from these studies suggest that the likelihood of TSD being relatively neutral compared to GSD in reptiles deserves more attention. Consequently, selection for female behavior and offspring thermal sensitivity to adaptively adjust sex ratios may be fairly weak. This conclusion is supported by the small observed differences in pivotal temperatures and lack of strong patterns of thermally-based nest-site selection.

# CHAPTER 1.

# GENERAL INTRODUCTION

## Dissertation Organization

This dissertation is organized in seven chapters. The first chapter provides a general introduction to sex ratio evolutionary theory, environmental sex determination, relevant information on the life history of painted turtles, and the objectives of this study. The second through sixth chapter are studies addressing the specific objectives outlined in the introduction. The second chapter evaluates the potential role of a nesting-specific behavior for detecting thermal cues for nest placement in female painted turtles. The third chapter assess variation in thermal sensitivity of offspring sex and yolk steroid hormones between two populations of painted turtles. The fourth chapter assesses variation in nesting behavior between these populations and its effects on nest temperatures. The fifth chapter evaluates the relative importance of these two traits, thermal sensitivity of sex and nest-site choice by females, in sex ratio evolution. The sixth chapter proposes alternative mechanisms for the evolution of environmental sex determination. The seventh and final chapter outlines the general conclusions from this dissertation.

## Introduction

> "In no case, as far as we can see, would an inherited tendency to produce both sexes in equal numbers or to produce one sex in excess, be a direct advantage or disadvantage to certain individuals more than to others; for instance, an individual with a tendency to produce more males than females would not succeed better in the battle for life than an individual with an opposite tendency;

and therefore a tendency of this kind could not be gained through natural selection...I formerly thought that when a tendency to produce the two sexes in equal numbers was advantageous to the species, it would follow from natural selection, but I now see that the whole problem is so intricate that it is safer to leave its solution for the future."

-Charles Darwin, 1874 *The Descent of Man*

One of the most important issues in biology is evaluating the role of adaptation in forming patterns of diversity among individuals, populations, and taxa. This emphasis stems from Darwin (1859), who first proposed descent by modification through natural selection as a major mechanism in forming patterns in organic structure and behavior. Darwin remained perplexed, however, by the evolution of sex ratios; why humans and domestic animals tended to produce male and female offspring in nearly equal numbers eluded him (Darwin, 1874). Ironically, Fisher (1930) explained that even this tendency could be attributed to a special form of natural selection, which was later known as frequency-dependent (or sex ratio) selection. Fisher's (1930) argument goes as follows: Since every offspring has exactly one female and one male parent, each sex supplies exactly half of the genetic contribution to the next generation. If one sex is rare, then it follows that its genetic contribution to the next generation is relatively greater (per capita) than that of the common sex. Any genetic tendency to produce the rarer sex will therefore increase in frequency in the next generation. As long as primary sex ratios (the sex ratio at conception) remain skewed and males and females have equal reproductive value, this process will continue across generations until primary sex ratios reach 1:1.

This view was later challenged by the observation that 1:1 sex ratios are constrained by Mendelian segregation of the sex chromosomes, rather than an adaptive consequence of frequency-dependent selection (Williams, 1979; Toro and Charlesworth, 1982). However, Fisher's (1930) principle was later upheld by its observation in a species of fish with environmental sex determination (ESD): when populations were reared in environments initially causing skewed sex ratios, balanced sex ratios eventually evolved (Conover and Van Voorhees, 1990). Fisher's principle has since been observed in experimental manipulations of *Drosophila* (Carvalho et al., 1998; Blows et al., 1999), confirming the importance of natural selection in sex ratio evolution.

On the other hand, 1:1 primary sex ratios have not been the norm for many non-mammalian species (Hamilton, 1967; Clark, 1978; Trivers and Willard, 1973; references in Charnov, 1982, Frank, 1989, and Hurst, 1993). Many of these cases include sex ratio distorters associated with sex chromosomes or cytoplasmic elements, which involve unequal transmission between the sexes (Hamilton, 1967; reviewed in Hurst et al., 1996). Other situations countering Fisherian sex ratio selection include competition among relatives for mates (Hamilton, 1967) or local resources (Clark, 1978). Additionally, offspring sex ratios may be adjusted according to the quality of resources provided by the parent or local environment; in other words, according to sex allocation strategy.

Sex allocation theory evaluates parental strategies for influencing offspring sex ratios, which is not necessarily the optimal sex ratio for the population. For example, in some polygynous mammals, females in good condition may bias offspring sex ratios toward sons because male offspring in good condition are thought to be more competitive in acquiring mates (Trivers and Willard, 1973). Since all female offspring are equally likely to

mate in this system, females in poor condition do better to invest in producing females of poor quality. In parasitoid wasps, females may adjust the sex ratio of their offspring according to host quality if high-quality hosts produce larger offspring, and one sex benefits most by large size (reviewed in King, 1987). In silverside fishes, reproductive success is related to body size in females but not for males; females tend to be born earlier than males, which allows more growing time before reproduction takes place (Conover, 1984). Do strategies for sex determination in these cases produce "optimal" sex ratios according to theoretical predictions? At least for silverside fishes, the answer appears to be "yes." Since this species exhibits a form of environmental sex determination (ESD) in concert with GSD, many individuals born in cool temperatures develop into females, and those born at warm temperatures tend to become males (Conover and Kynard, 1981). Most females are born during cool spring temperatures and have a long growing season before reaching reproductive maturity, whereas males are produced later in the season during warmer temperatures. In terms of a general conclusion, however, the question of whether sex ratios are a nonadaptive consequence of the sex determining system needs to be considered (Williams, 1979).

With the exclusion of silverside fishes and a few invertebrates, the evolutionary significance of ESD has been particularly troubling in this aspect. In this sex-determining mechanism, an individual's sex is affected by the environmental conditions in which it develops, rather than by sex chromosomes. A variety of environmental factors are known to affect sex ratios in invertebrates, including nutritional provisioning, density, photoperiod, or location of potential mates (reviewed in Korpelainen, 1990). Temperature affects sex in silverside fishes (Conover and Kynard, 1981), and strongly determines sex in many reptiles

(reviewed in Bull, 1980). Despite its widespread presence, ESD defies explanation in light of Fisher's principle of sex ratio selection. The reason for this was demonstrated mathematically by Bull (1983): when the environment varies temporally, primary sex ratios of an ESD population will vary accordingly over time. For example, in silverside fishes, due to among-year climatic variation, hotter years will overproduce males and cooler years will overproduce females. Bull (1983) then showed that as long as environmental conditions do not produce exactly a 1:1 sex ratio each year, ESD experiences a selective disadvantage to GSD according to Fisherian sex ratio selection. In this vein, ESD has been generally considered an adaptation to particular life histories (Charnov and Bull, 1977) to account for its otherwise maladaptive persistence.

Charnov and Bull's (1977) model laid out the series of specific conditions necessary for ESD to be adaptive: (1) environmental conditions are spatially variable, (2) offspring and parents have no control over which environmental condition ("patches") the offspring encounters, (3) parents mate randomly with respect to the patches that they came from, and (4) some patches enhance male fitness greater than female fitness, and vice-versa in other patches. In this situation, an individual with genotypic sex determination (GSD) will be at a disadvantage because it is likely to develop into the sex experiencing lower fitness for that patch type. Individuals with ESD, however, would always become the "right" sex for the given patch type. Although the Charnov-Bull model clearly makes sense in light of silverside fishes and a few sessile invertebrates (Bull, 1983), the lack of convincing experimental evidence supporting condition (4) in reptiles has frustrated attempts to explain its evolution and persistence. Hence, the adaptive significance of this form of ESD,

temperature-dependent sex determination (TSD) has been the subject of ongoing debate for more than two decades (reviewed in Shine, 1999).

**Models for the adaptive significance of TSD in reptiles.**

Although TSD exists in all crocodilians, most turtles, and several lizards, patterns of TSD vary such that temperatures producing males in some taxa produce females in others (Bull, 1980; Paukstis and Janzen, 1990; Figure 1). For example, warm incubation temperatures produce females and cool temperatures produce males in most turtles, whereas the opposite pattern occurs in some lizards and most crocodilians. In some turtles and crocodilians, intermediate temperatures produce males and extreme temperatures produce females. Again, this diversity in patterns of TSD has become a source of speculation for its adaptive value, but has not elicited a satisfactory explanation (Bull and Charnov, 1989; Ewert and Nelson, 1991; Janzen and Paukstis, 1991b).

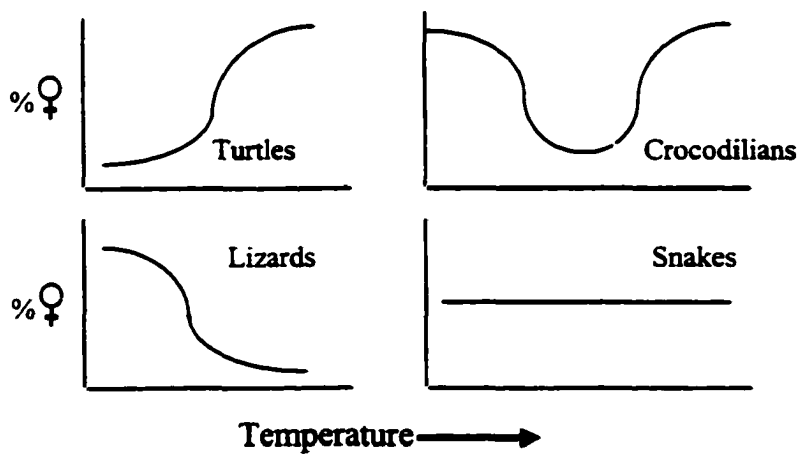


**Figure 1.** Common patterns of temperature-dependent sex determination in reptiles. Sex ratio (% females) as a function of increasing incubation temperatures. Figure adapted from Bull (1980).

There are three major theories concerning TSD as an adaptation to life histories in reptiles (for reviews see Ewert and Nelson, 1991, Burke, 1993, and Shine, 1999). First, TSD may be a mechanism that prevents inbreeding among siblings from the same clutch (Ewert and Nelson, 1991). Many natural nests of species with TSD are unisexual, since typically an extremely narrow range of temperatures produces mixed sex ratios (Ewert and Nelson, 1991). Obviously, siblings from the same clutches cannot mate if they are all of the same sex. However, Ewert and Nelson (1991) note that this mechanism does not prevent inbreeding between parents and offspring, or between half-siblings for that matter. Furthermore, one must demonstrate that the fitness cost of inbreeding for reptiles with TSD is so great compared to reptiles with GSD that it warrants a completely different sex determining mechanism. Given the onerous assumptions it entails, this hypothesis has generally not been viewed as a likely mechanism for the evolution of TSD (Burke, 1993; Shine, 1999).

A second hypothesis for the adaptive significance of TSD concerns group-structured adaptation. In small populations of related individuals, non-Fisherian sex ratio allocation is sometimes favored (e.g., Hamilton, 1967). For example, the apparent female-biased hatchling sex ratios observed across some populations of TSD reptiles may be explained if male siblings compete for mates (Ewert and Nelson, 1991). However, even small amounts of exchange among subdivided populations causes masculinizing alleles to invade a population favoring skewed sex ratios, and genetic as well as life history data do not support a lack of such exchange for reptiles with TSD (Burke, 1993). Furthermore, these two aforementioned hypotheses simply explain adaptive sex ratio skewing in general, but do not

offer an explanation for the evolution of TSD as a sex-determining mechanism in particular (Burke, 1993; Shine, 1999).

Finally, the most widely accepted hypothesis follows condition (4) of the Charnov-Bull (1977) model: some environmental parameter affects offspring fitness differentially according to sex (reviewed in Burke, 1993 and Shine, 1999). Since the Charnov-Bull model seems the logical explanation for the evolution of ESD in other taxa (Bull, 1983), perhaps reptiles also experience a "differential fitness" between the sexes according to their incubation conditions. Convincing evidence for such a differential fitness effect, however, has not been established in over 25 years of study since the model had first been proposed (reviewed in Shine, 1999). Considering the longevity of most reptiles with TSD, measuring the lifetime reproductive success of any individual would be a formidable task. Therefore, investigations have focused primarily on hatchling phenotypes that are expected to be correlated with fitness (e.g., growth rates: Joanen et al.,1987; anti-predator behavior: Janzen, 1995; mate choice: Shine et al., 1995). This focus produces several major problems, however. Since the studies vary in which phenotypic traits were measured, forming a general mechanism pertaining to all TSD taxa is impossible at this time. Furthermore, in practice, the differential fitness hypothesis cannot be rejected using this method: if it fails to be observed for one phenotypic trait, it could conceivably be upheld in another, unmeasured trait possibly correlated with fitness (see Brown, 2001 for this argument concerning the Trivers-Willard hypothesis).

In fact, not only do measured traits vary among studies, but also the underlying mechanisms allowing each sex to differentially benefit from temperature. Shine (1999) summarizes six of these differential fitness models that have been applied to reptiles with

TSD, with varying degrees of success (e.g., ESD as a correlate of seasonal timing of hatching, to allow maternal matching of sex to offspring size, etc.). Again, these models appear to be taxon-specific. For example, if TSD allows male lizards to hatch earlier than females so males can grow to be larger in time for reproduction (Harlow and Taylor, 2000), this two-week difference in hatching time is insignificant for turtles or crocodiles, which mature several years after hatching. This continuous search for potential traits and mechanisms brings to mind the classic scenario central to Gould and Lewontin's (1979) criticism of the adaptationist programme: "if one adaptive argument fails, try another." Given the variation of patterns of TSD among taxa and the logistic difficulties of testing the Charnov-Bull model in reptiles, evidence generally supporting or clearly rejecting the model will likely remain elusive for quite some time.

**Models suggesting that TSD may not be adaptive in reptiles.**

Although the aforementioned adaptive models may explain how TSD may be maintained, they do not necessarily imply that TSD evolved by the same mechanism. Taxa exhibiting TSD are ancient in origin (Janzen and Paukstis, 1988; 1991a,b), and conditions that may have been adaptive in the past have likely changed. However, the only model explaining how TSD may evolve from GSD invokes the Charnov-Bull "differential fitness" condition (Bull, 1981). A second major hypothesis is that TSD may in fact not be adaptive, and simply persists as an ancestral form of sex determination that has not undergone a strong selective disadvantage (Janzen and Paukstis, 1988; Ewert and Nelson, 1991; Burke, 1993; Shine, 1999). Two key observations may shed light on the viability of this "phylogenetic

inertia" hypothesis: the status of TSD as an ancestral mechanism in vertebrates, and its relative disadvantages compared to GSD in reptiles.

Unfortunately, whether TSD or GSD was ancestral in amniote vertebrates is unclear (Janzen and Paukstis, 1991). TSD is ubiquitous in crocodilians and almost certainly basal in chelonians, but GSD predominates in lizards and is present in all snakes (Janzen and Paukstis, 1991). To confuse matters further, the tuatara exhibits TSD (Cree, 1995), suggesting a pleisiomorphic status in squamates. On the other hand, GSD is known in all amphibians to date (Hayes, 1998) and exists in all birds and mammals (reviewed in Bull, 1980). Although its evolutionary status remains debatable, Shine (1999) recently disregarded the possibility that TSD lacks adaptive significance because of the phylogentic lability of sex determining mechanisms in reptiles. However, by the same argument, one could propose that its phylogenetic lability suggests that TSD is more or less neutral compared to GSD.

Indeed, TSD may suffer little adaptive "disadvantage" compared to GSD, considering the longevity of most reptiles exhibiting TSD. In iteroparous species with populations exhibiting large numbers of overlapping generations, an individual's reproductive success is based on the primary sex ratio of all reproducing cohorts rather than on the primary sex ratio of its own cohort (Girondot and Pieau, 1996). When environmental conditions vary temporally but fluctuate around a stable mean, this weighted average of all cohort primary sex ratios will be buffered from extreme sex ratio bias (Girondot and Pieau, 1996). Longer life spans also increase the likelihood that these fluctuations in offspring sex ratios will cancel out over the lifetime of an individual with ESD (Bull and Bulmer, 1989), putting ESD on a similar playing field with GSD in terms of Fisherian sex ratio selection.

This scenario was not initially considered by Bull (1983), who used only non-overlapping generations in his model comparing the selective advantage of GSD over ESD in temporally variable environments. However, when Bull and Bulmer (1989) considered this effect of longevity on reducing potential disadvantages of ESD, they concluded that longevity instead enhances selection for ESD! They argued that longevity requires only a small differential fitness advantage for ESD to be maintained (Bull and Bulmer, 1989). Girondot and Pieau (1999) instead suggest that longevity would then allow ESD to be maintained for long periods of time as a completely neutral sex determining mechanism with no selective advantage. Their argument also implies that ESD may be neither pleisiomorphic nor adaptive, a distinct possibility that has never been explicitly proposed. In sum, it appears that arguments for or against the adaptive significance of TSD in reptiles tend to draw opposite conclusions from similar data.

**Given that TSD exists in reptiles, how do sex ratios evolve?**

Theories concerning the adaptive significance of TSD in reptiles do not necessarily explain why this sex determining system has continually persisted through environmental change. For example, a differential fitness benefit cannot give TSD a selective advantage over GSD if the environment has changed to the extent that one sex will no longer be produced. How sex ratios evolve under TSD is particularly intriguing in reptiles because thermal incubation differences as small as 1.5 – 2 °C can determine whether 100% males or 100% females are produced in both field and laboratory conditions (Bull 1980; Bull 1985; Ewert and Nelson 1991; Paukstis and Janzen, 1990; Janzen 1994a; Weisrock and Janzen 1999). In fact, one long-term study documents a high correlation between climatic condition

during the egg incubation season and annual primary sex ratio in a freshwater turtle with TSD: an increase in climate as small as 2°C is expected on average to produce 100% females in the population (Janzen, 1994a). Seasonal climatic changes similarly skew primary sex ratios within a year for some sea turtles (Mrosovsky, 1984).

Once TSD is established, sex ratios are thought to evolve through heritable variation in and selection on two main mechanisms: thermal sensitivity of offspring sex, and nesting behavior by females affecting nest thermal conditions (Bulmer and Bull, 1982). Variation in the first mechanism has been widely documented in turtles and some crocodilians with TSD. When eggs are incubated in the laboratory at a constant temperature expected to produce an overall 1:1 sex ratio in the population (called the pivotal temperature), different families exhibit a strong tendency to produce mostly males or females (Bull et al., 1982a; Ewert et al., 1994; Janzen, 1992; Rhen and Lang, 1998). However, pivotal temperatures have been thought to have low potential to evolve in the wild because variation in nest temperatures easily exceeds the narrow thermal range wherein genetic variation for sex ratio is expressed (Bull et al., 1982a; Bulmer and Bull, 1982; Janzen, 1992). In other words, the thermal environment has a relatively greater effect on sex ratio: any given nest is expected be placed in thermal environments producing either all males or all females, regardless of the genetic predisposition of that family for sex ratio. Geographic patterns of sex determination indeed reflect a low likelihood for pivotal temperatures to evolve adaptively within species. For example, it may be expected that higher pivotal temperatures occur in lower latitudes in North American turtles so that more males will be produced in hotter climates. However, latitudinal clines in pivotal temperatures have generally been either lacking or in the

opposite direction expected (Bull et al., 1982b; Mrosovsky, 1988, 1994; Vogt and Flores-Villela, 1992; Ewert et al., 1994).

For these reasons, nesting behavior by females has generally been accepted as a more plausible scenario for how sex ratios may evolve and be maintained in reptiles with TSD (Bull et al., 1982a; 1982b; Bulmer and Bull, 1982; Vogt and Bull, 1982; Ewert et al., 1994; but see Rhen and Lang, 1998). Perhaps nest microclimates are similar across a species range: females could place eggs in relatively cool microenvironments in hotter climates, and in relatively hot microenvironments in cold climates. Since sex determination typically takes place several weeks after a nest is laid (Schwarzkopf and Brooks, 1985; Janzen, 1994b), variation in nest-site selection may be based on lasting environmental cues correlated with nest temperatures several weeks after oviposition. Such cues include vegetation cover over nests (Vogt and Bull, 1984; Janzen, 1994b; Roosenburg, 1996; Wilson, 1998; Weisrock and Janzen, 1999), slope or aspect of nests (Schwarzkopf and Brooks, 1987), nest depth (Wilhoft et al., 1983), or sand albedo (Hays et al., 2001). In addition to nest-site choice, timing of nesting is another behavior that may affect nest temperatures and consequently sex ratios (Mrosovsky, 1984). Similar to some populations of birds (Dunn and Winkler, 2000) and amphibians (Beebee, 1995), timing of reproduction may also be correlated with climatic condition in reptiles. However, geographic variation in nesting patterns in reptiles with TSD has remained largely uninvestigated, with the exception of a few casual observations. For example, Mexican red-eared slider turtles (*Trachemys scripta*) tend to lay eggs in more heavily vegetated areas (Vogt and Flores-Villela, 1992), whereas their northern conspecifics tend to oviposit in open areas (Cagle, 1950; Ernst et al., 1994; Tucker, 1997). Some North American turtles exhibiting TSD nest

earlier in southern latitudes than in northern latitudes (Chippindale, 1989; Ernst, 1970; Ernst et al., 1994; Ewert, 1976; Robinson and Bider, 1988), but again, how such variation may affect nest temperatures in these species has not been documented.

**The painted turtle, a reptile with temperature-dependent sex determination**

The painted turtle (*Chrysemys picta*) is one of the most widely distributed freshwater turtles in the world. It ranges across the northern United States from Washington to Maine and through central North America from southern Ontario to Louisiana (Ernst et al., 1994). In particular, the western painted turtle (*C. picta bellii*) has the widest latitudinal distribution among the four subspecies that have been described, ranging from Manitoba to Texas (Conant and Collins, 1991). Despite having such a widespread geographic range, offspring sex ratios of painted turtles are extremely sensitive to temperature. For example, 98% males were produced when *C. picta bellii* eggs from Wisconsin were incubated at a constant temperature of 28°C, whereas 100% females were produced from the same population when incubated at 29.5°C (Bull et al., 1982b). Similarly, *C. picta bellii* primary sex ratios from field-incubated eggs are highly correlated with climatic condition during the period of egg incubation for a natural population in Illinois (Janzen, 1994a). Hot summer temperatures during July 1988 produced 100% female offspring for that year, but 100% males were produced during 1992, which experienced cool July temperatures (Figure 2). Janzen (1994a) also observed that July air temperatures experienced at that field site for the past 50 years are expected on average to produce nearly a 1:1 sex ratio in that population.

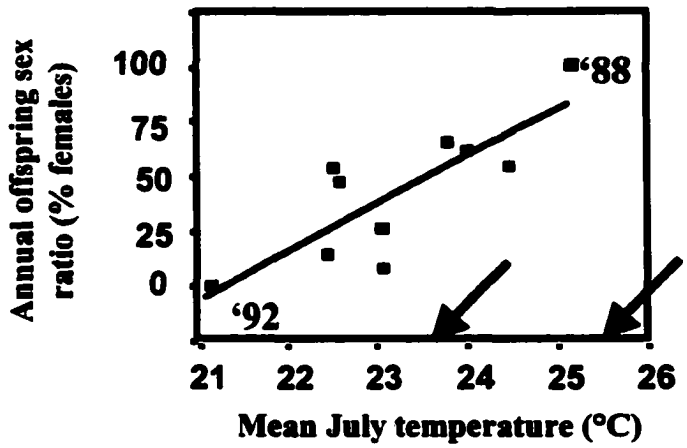


**Figure 2.** Annual cohort sex ratios as a function of July temperature in a natural population of painted turtles (*Chrysemys picta*) in Thomson, Illinois. Figure is based on Fig.1 in Janzen (1994a). The left arrow indicates the 50-year average July temperature for Thomson, Illinois (Janzen, 1994a), and the right arrow indicates the 30-year average July temperature at a thriving *C. picta* population at the Bosque del Apache National Wildlife Refuge, New Mexico (National Oceanic and Atmospheric Administration, 2000).

In New Mexico, mean July air temperatures experienced at locations of thriving *C. picta bellii* equal those expected to produce 100% females in Illinois (Figure 2; National Oceanic and Atmospheric Administration, 2000). Do these populations experience highly female-biased sex ratios, or do major differences in life history traits exist between New Mexico painted turtles and their northern conspecifics? Unfortunately, very little life history information is known on *C. picta* in New Mexico (Degenhardt et al., 1996). Timing of egg production is known to be similar between western painted turtles located in New Mexico and Wisconsin (Christenson and Moll, 1973), suggesting that nesting dates are also likely to be similar among *C. picta* populations. Given that no previous studies have quantified differences in nesting ecology among populations of reptiles with TSD, and the large amount of data available for Illinois *C. picta* (e.g., Janzen 1994a, 1994b; Weisrock and

Janzen, 1999; Janzen and Morjan, 2001; Valenzuela and Janzen, 2001), *C. picta* populations located in New Mexico provide a valuable comparison for evaluating potential mechanisms for sex ratio evolution.

**Objectives**

This research involves five projects designed to evaluate potential mechanisms for the maintenance and evolution of environmental sex determination. In all papers, the western painted turtle is used either as a study organism or as the basis for key life history information incorporated into theoretical models. The specific objectives are to:

(1) evaluate a specific nesting behavior as a potential mode for females to discriminate among thermal nesting environments.

(2) evaluate differences in pivotal temperature between a northern and southern population of painted turtles as a potential mechanism for maintaining sex ratios.

(3) ascertain and compare patterns of nest-site choice in the two populations as a mechanism that maintains similar nest temperatures.

(4) predict the relative influence of pivotal temperatures and nest-site choice in the evolution of sex ratios in this system, based on life history information of these turtles.

(5) develop alternative models to the Charnov-Bull hypothesis for the evolution of environmental sex determination.

# CHAPTER 2.

## Is Ground-nuzzling by Female Turtles Associated with Soil Surface Temperatures?

A paper published in the Journal of Herpetology[1]

Carrie L. Morjan[2,3,4] and Nicole Valenzuela[2]

Department of Zoology and Genetics, Iowa State University

Ames, IA 50011

Nest-site choice in reptiles has elicited considerable interest because of its implications for offspring fitness. Nest location may directly affect offspring survivorship, in terms of predation risk, hatching success, and emergence success (Whitmore and Dutton, 1985; Temple, 1987; Horrocks and Scott, 1991; Wilson, 1998). Environmental variation among oviposition sites, such as temperature and hydric conditions, influences survivorship and various offspring traits including sex, growth, and behavior (Bull 1980; Gutzke and Crews, 1988; Packard et al., 1993; Shine and Harlow, 1996; reviewed in Deeming and Ferguson, 1991). Given these consequences of incubation conditions on offspring survivorship and phenotype, selection may exist for females to detect environmental cues correlated with offspring fitness and to oviposit at sites exhibiting these cues (see Resetarits, 1996).

This study assesses the role of ground temperature as a cue for nest-site choice in painted turtles (*Chrysemys picta*). In painted turtles, temperatures in natural nests affect offspring sex (Schwarzkopf and Brooks, 1987) and survivorship (Packard et al., 1997;

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[1]Reprinted with permission of J. of Herpetology, 2001, 35(4), 668-672.

[2]Graduate student and Associate Scientist/Adjunct Professor, respectively, Department of Zoology and Genetics, Iowa State University

[3]Primary researcher and author.

[4]Author for correspondence.

Weisrock and Janzen, 1999). In some evolutionary models, the ability of females to discriminate among nesting environments with different thermal characteristics is a critical assumption for the maintenance of temperature-dependent sex determination (Roosenburg, 1996). We specifically focused on a behavior present in many turtles that is thought to aid in nest-site choice through the detection of environmental cues. Female turtles frequently exhibit a stereotypical behavior during nesting forays, in which the head and ventral side of the neck are pressed on the ground surface before choosing a nest site. This so-called "ground-nuzzling" or "sand-smelling" is particularly interesting because of its presence across a wide array of chelonian taxa (Table 1), and may be a conserved mechanism for detecting temperature, olfactory, or other cues during nest-site selection (Carr et al., 1966; Ehrenfeld, 1979; Stoneburner and Richardson, 1981). No study has investigated the correlation between this behavior and any potential environmental cue. Because it is uncertain whether this behavior is in fact an olfactory sensory mechanism, and because it occurs not only in sand, we will refer to it as ground-nuzzling rather than sand-smelling.

We observed natural nesting events of painted turtles to examine whether surface temperatures experienced by females while ground-nuzzling differed from surface temperatures at nesting or walking. We further evaluated the potential utility of surface temperature as a cue to nesting females by investigating whether surface temperatures at the time of nesting were correlated with vegetation cover over nests (a known thermal indicator at this site), sex ratios, predation rates, and hatching success of nests.

We observed nesting painted turtles (*C. picta bellii*) on the south nesting beach at the Thomson Causeway Recreation Area in Thomson, Illinois (41°57'N, 90°07'W). The nesting beach is level and fairly uniform in soil moisture levels, and trees are dispersed across the

site, providing a wide range of thermal environments for nest placement (Janzen, 1994). This site is a public recreation area, and nesting turtles consequently have become fairly tolerant to human observation. Forty-seven turtles were observed following their emergence onto the nesting beach between 28 May and 30 June 1998. During this time, we searched the nesting beach for females at least once hourly from sunrise until sunset. Each turtle was observed from the time she was first discovered (usually shortly after emergence from the water) until she either commenced nesting or left the nesting beach and returned to the water. Most turtles emerged during the afternoon (1400 to 2000 h), although some emerged in the morning (0700 to 1000 h).

We chose to measure ground temperatures at fixed time intervals rather than at fixed distances during the turtles' nesting forays, because females rarely walked a straight path during their nesting forays while covering a long distance from the water to the nest. Furthermore, no tracks were left by the turtles as they traveled over the short grass of the nesting beach, and marking the turtle's path would likely interfere with normal nesting behavior. We observed turtles by standing motionless at a vantage point 5-10 m away from the turtle during her nesting foray. Surface temperatures were measured with a factory-calibrated Raytek® Raynger ST8 non-contact, infrared thermometer (accuracy ± 1°C). This thermometer functions by pointing a sighting laser directly at a surface and measuring the temperature of the surface remotely from the point of the laser. This instrument has a 30:1 distance-to-spot ratio, meaning that at a 10-m distance the instrument records surface temperatures from a circle with a 33-cm diameter, centered on the laser point visible on the ground's surface. Temperatures were measured every 30 seconds during the turtle's nesting foray by pointing the infrared thermometer directly at the ground surface in front of the

turtle's nose. We aimed the sighting laser at a short distance in front of the turtle (10 - 20 cm) in an effort to measure the ground's surface temperature while excluding the turtle's temperature from the measurements as much as possible. If the turtle stopped moving for > 1 minute, recording was stopped until she resumed movement.

Temperature and the behavior of the turtle were registered into a tape recorder. Four behaviors were identified: "walking," "standing," "ground-nuzzling," and "nest digging." We recorded every time the female performed the ground-nuzzling or nest digging behavior, such that the remaining time corresponded to walking or standing which were thus combined into a third behavioral category (walking/standing). These three behavioral categories were considered for statistical analysis. When a female commenced "nest digging," she usually initiated this behavior by turning 180° immediately after a "ground-nuzzling" episode, and digging at this site with her hind legs. However, females frequently exhibited the ground-nuzzling behavior at several sites during their nest-site selection process before nesting at the final site.

In order to exclude repeat nesters (maximizing the independence of the data), turtles were identified on completion of nesting by examining unique patterns of notches on the marginal scutes, made during previous studies at the site. If no marks were present, she was given a new marking pattern. Eggs from nests were excavated, counted, and reburied in their original orientations. Vegetation cover, the amount of canopy vegetation over the nest, was recorded over each nest while facing each of the four cardinal directions with a Model-A spherical densiometer as in Janzen (1994). This variable was considered important to measure because within each nesting season, nest temperatures and offspring sex ratios are correlated with vegetation cover from the south and west directions over nests (Janzen,

1994; Weisrock and Janzen, 1999; Morjan and Janzen, unpubl. data). Air temperatures were recorded at the field site every 40 min from 30 May until the end of the study using a HOBO TEMP® datalogger placed out of direct sunlight at 0.3 m above the ground (Weisrock and Janzen, 1999).

In early September 1998, hatchlings were removed from 14 of the 15 nests that survived predation. The remaining nest was part of an ongoing study concerning overwinter survivorship and these hatchlings were removed in March 1999. Hatching success was defined as the number of hatchlings divided by the number of eggs in an intact nest. Up to 6 hatchlings per nest were euthanized by injection of a 1:1 solution of sodium-pentobarbital ("Sleepaway") and deionized water into the cardiac cavity. Hatchlings were sexed by macroscopic examination of the gonads and preserved in ethanol in the laboratory at Iowa State University.

We first determined whether the relationship between air temperature (taken within 20 minutes of surface temperature measurements) and surface temperature was homogeneous for all nesting forays. An analysis of covariance using female identity as an effect, air temperature as the covariate and surface temperature as the response variable showed significant differences among females (equal slopes but different intercepts). Therefore, all the analyses that follow were conducted taking into account female identity. Residuals of the regression of surface temperature on air temperature used in the previous analyses were tested for the presence of serial autocorrelation using the Durbin-Watson test (Neter et al., 1996). We corrected for this serial autocorrelation by performing a three-way Mantel test (Smouse et al., 1986) of residual temperature by behavioral category ("walking/standing," "ground-nuzzling," or "nesting"), holding time and female constant. We generated four

distance matrices corresponding to the time, behavioral category, turtle ID and residual temperature variables. We then combined the time and turtle ID matrices into a new design matrix where time values were kept only in those cells corresponding to same-female comparisons, and all other cells corresponding to comparisons across females were given a value of zero. By performing the three-way Mantel test of residual temperature by behavioral category holding this new design matrix constant, we tested for differences in temperatures among behavioral categories while simultaneously holding the effects of time and female constant.

Finally, we tested whether surface temperature at nesting was correlated with vegetation cover over the nest, nest predation, hatching success, and hatchling sex ratios. Residuals were taken from the regression of air temperatures on surface temperatures at time of nesting. Percent vegetation cover was calculated by converting the values taken from the south and west densiometer readings into percentages and calculating their average. We performed a linear regression of residual temperature of the surface over the nest site at time of nesting on percent vegetation cover over the nest. Then we performed a series of logistic regression analyses to investigate the effect of residual temperature on nest predation (N = 17 nests), hatching success (N = 12 nests), and sex ratios (percent females) for nests producing hatchlings (N = 12 nests).

Of the 49 turtles observed during the study, 26 successfully constructed nests. The ground-nuzzling behavior was significantly associated with nesting (Fisher's Exact test; P = 0.0002). Only one of the turtles that nested was not observed to exhibit ground-nuzzling. Of the 23 turtles that did not nest, 12 were never observed to exhibit the ground-nuzzling behavior, four were frightened from nesting due to known disturbances unrelated to the

study, and three abandoned nest cavities for unknown reasons. Based on these observations, and given an incidence of false crawls (emergence without nesting) up to 71% in some nesting turtle populations (Andre and West, 1981), we are confident that observing the females in this population did not cause disturbance to their normal nesting behavior.

Using ANCOVA, we found a significant relationship between air temperature and surface temperature, which was homogeneous among females (parallel slopes, $F = 0.960$, $df = 39$, $P = 0.5423$). Residual ground temperatures were serially autocorrelated (Durbin-Watson index = 0.58, $P < 0.001$). No significant differences among behavioral categories in residual ground temperatures were evident, even while accounting for female effects and serial autocorrelation ($P = 0.66$).

Air temperatures and surface temperatures at nesting were highly correlated ($N = 17$, $R^2 = 0.53$, $P = 0.001$), so residual ground temperatures were used to assess the association between surface temperatures at nesting and nest characteristics. Residual ground temperatures at nesting were uncorrelated with the percentage of vegetation cover over the nest ($N = 17$, $R^2 = 0.06$, $P = 0.35$). Although vegetation cover over nests was not correlated with hatchling sex ratio ($N = 14$, $\chi^2_{(1)} = 2.37$, $P = 0.12$), there was a significant relationship between residual ground temperatures at nesting and sex ratio such that relatively warmer sites tended to produce more females ($N = 12$, $\chi^2_{(1)} = 7.65$; $P < 0.006$; $R^2 = 0.09$,). Residual ground temperatures had no significant effect on whether a nest was depredated ($N = 17$, $\chi^2_{(1)} = 0.16$, $P = 0.69$) or on the hatching success of a nest ($N = 12$, $\chi^2_{(1)} = 0.72$, $P = 0.40$). All analyses using unstandardized ground temperatures in the place of residual ground temperatures were not significant.

In conclusion, we found that ground-nuzzling in *C. picta* was significantly associated

with nesting, but no differences in ground temperature existed among the three behavioral categories (standing/walking, ground-nuzzling and nesting). If females select nest sites based on thermal cues, it might be expected that temperatures at the nest site should differ from those encountered prior to nesting during the search for a nest site. Stoneburner and Richardson (1981) reported that female loggerhead turtles (*Caretta caretta*) nested when they encountered an abrupt increase in temperature (>2.0 °C) on the nesting beach. They speculated that the ground-nuzzling behavior exhibited in this species may act as a possible mechanism to detect such thermal cues. However, Wood and Bjorndal (2000) noted the observed thermal differences may have been artifactual (overturned sand during the nest digging process may have influenced the results), and that other characteristics such as slope may instead influence nest-site choice. Our results showed no consistent significant thermal differences among the behavioral categories studied among females; thus, females are unlikely to be choosing warmer sites for oviposition based on surface temperatures, as found by Stoneburner and Richardson (1981).

Ground temperatures at nesting for *C. picta* did not differ overall from temperatures encountered during the nesting foray in this study. These results suggest that females either do not use surface temperatures as a cue for nesting, or that females may in fact choose to nest at sites exhibiting a broad range of intermediate temperatures and simply avoid areas exhibiting extreme temperatures. Nests of *C. picta* in Ontario experience significantly warmer temperatures than random sites, and females probably use slope and absence of vegetation as cues to select their nesting site (Schwarzkopf and Brooks, 1987). With this possibility in mind, we investigated potential relationships between vegetation cover, surface temperatures, survivorship, and hatchling sex ratios. We did not consider slope in

these analyses because the nesting beach was uniformly level. We found a negative, but not significant relationship, between vegetation cover and proportion of female hatchlings for the focal females in this study. Although the trend was in the expected direction, our small sample size for this analysis (N = 14 nests) is likely responsible for the weak association between these two variables. Indeed, vegetation cover significantly affected hatchling sex ratios for the entire study site in 1998 (F. J. Janzen, pers. comm.). However, we did find a positive and significant relationship between the residual ground temperatures at nesting and sex ratio, but this correlation had a very low predictive power ($R^2 = 0.09$). Despite the fact that vegetation cover negatively affects offspring sex ratios in this population and that relatively warmer sites at nesting also positively affected sex ratios, we found no significant relationship between the surface temperatures at nesting and vegetation cover over the nest with our sample size.

The ground-nuzzling behavior may perhaps be related to the detection of olfactory cues. Hatchling *Lepidochelys kempi* preferentially oriented to seawater that was in contact with substrate from where they were incubated and hatched (Grassman et al., 1984). If hatchlings imprint to chemical properties of the incubation and hatching substrate, and natal philopatry exists, ground-nuzzling behavior in nesting females could act as a mechanism to detect these chemical properties when returning to natal beaches. Carr and Hirth (1962) observed that female green turtles (*Chelonia mydas*) exhibited the ground-nuzzling behavior in shallow water even before emerging from the surf, and that the tip of the snout, rather than the entire neck, was placed on the sand. The ground-nuzzling behavior was usually terminated long before the nest-site selection process was completed for females of this species (Table 4 in Carr et al., 1966). At least for green turtles, it would be difficult to

explain why this behavior might be used to detect temperature rather than olfactory cues for emerging at a particular nesting beach. *Chrysemys picta* females are philopatric to nesting areas (Schwarzkopf and Brooks, 1987; Scribner et al., 1993); thus it is possible that they employ ground-nuzzling to aid in philopatry.

One important result from our study is that our data allowed us to rule out temperature as a factor related to ground-nuzzling. It is quite surprising that this behavior has received such little attention despite being widespread among chelonian taxa (Table 1). Further studies should test whether this behavior is instead an olfactory sensing mechanism.

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**Table 1.** Turtles reported to exhibit the "ground-nuzzling" behavior.

| | |
|---|---|
| *Cryptodira* | |
| Cheloniidae | |
| *Caretta caretta* | (Stoneburner and Richardson, 1981) |
| *Chelonia mydas* | (Carr and Hirth, 1962) |
| *Eretmochelys imbricata* | (Carr et al., 1966) |
| *Lepidochelys* | (Hirth and Carr, 1970) |
| Emydidae | |
| *Chrysemys picta* | C. Morjan and N. Valenzuela, pers. obs. |
| *Clemmys guttata* | (Ernst et al., 1994) |
| *Clemmys insculpta* | (Harding and Bloomer, 1979) |
| *Clemmys muhlenbergii* | (Holub and Bloomer, 1977) |
| *Emydoidea blandingi* | (Linck et al., 1989) |
| *Graptemys ernsti* | (Ernst et al., 1994) |
| *Graptemys nigrinoda* | (Ernst et al., 1994) |
| *Malaclemys terrapin* | (Lazell and Auger, 1981) |
| *Pseudemys concinna* | (Jackson and Walker, 1997) |
| *Terrapene carolina* | (Messinger and Patton, 1995) |
| *Trachemys gaigeae* | (Morjan and Stuart, in press) |
| *Trachemys scripta* | (Moll and Legler, 1971) |

**Table 1.** (Continued)

| | |
|---|---|
| Testudinidae | |
| *Testudo hermanni* | (Swingland and Stubbs, 1985) |
| *Pleurodira* | |
| Pelomedusidae | |
| *Podocnemis expansa* | N. Valenzuela, pers. obs. |

# CHAPTER 3.

## Variation in offspring sex ratios and yolk steroid hormone concentrations in two populations of painted turtles (*Chrysemys picta*) with temperature-dependent sex determination.

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Carrie L. Morjan

Department of Zoology and Genetics, Iowa State University

Ames, IA 50011

## ABSTRACT

Sex ratios in reptiles with temperature-dependent sex determination (TSD) are thought to evolve and be maintained in part by genetic variation in pivotal temperatures (constant temperature producing a 1:1 sex ratio). In this study, I compared pivotal temperatures between two geographically separated populations of western painted turtles (*Chrysemys picta bellii*) located in New Mexico and Illinois, and quantified yolk estradiol and testosterone concentrations from both populations as a potential source for differing pivotal temperatures. In accordance with theory, eggs from New Mexico exhibited a significantly higher pivotal temperature (producing more males at hotter temperatures) than those from Illinois. However, 1:1 sex ratios were not produced when eggs were incubated at these calculated pivotal temperatures during a subsequent nesting season, possibly due to seasonal effects on sex determination for both populations. Such a seasonal effect on sex determination (more males produced earlier) was detected in the Illinois population. Eggs from New Mexico exhibited higher yolk estradiol concentrations and estradiol:testosterone

(E:T) ratios than those from Illinois, opposite to the direction expected from known effects of estradiol on sex determination in *C. picta*. These results suggest that sex ratios in these populations may in part be maintained by geographic variation in pivotal temperatures, but are not readily explained by among-population variation in yolk estradiol concentrations.

## Introduction

Many reptiles exhibit temperature-dependent sex determination (TSD), a sex determining mechanism in which egg incubation temperatures, rather than sex chromosomes, permanently determine offspring sex. Although Mendelian segregation of the sex chromosomes tends to ensure nearly 1:1 sex ratios in vertebrates with genotypic sex determination (GSD), is not clear in reptiles with TSD how sufficient frequencies of both sexes may be maintained under changing thermal conditions. Genetic variation and selection on two main traits are thought to allow sex ratios to evolve and be maintained in this system: (1) female nesting behavior affecting nest temperatures, and (2) sex ratio of embryos in response to incubation temperatures (Bulmer and Bull, 1982). Empirical studies to date have mainly focused on within- and among-population variation in the latter of these two traits (Bull et al., 1982a; Bull et al., 1982b; Janzen, 1992; Vogt and Flores-Villela, 1992; Ewert and Nelson, 1994; Rhen and Lang, 1998).

Most assessments of phenotypic variation in sex determination in reptiles with temperature-dependent sex determination (TSD) assume that observed differences in sex ratios among families are due mainly to genetic causes when eggs are incubated at constant temperatures in the laboratory (Bull et al., 1982a; Bull et al., 1982b; Janzen, 1992; Rhen and Lang, 1998). However, the recent discovery of seasonal variation in sex determination due

to varying concentrations of maternally derived yolk steroid hormones in a turtle with TSD (Bowden et al., 2000) raises the possibility that nongenetic maternal effects may have an important role in sex determination. Bowden et al. (2000) reported a seasonal decrease in the proportion of males produced throughout each of five separate incubation seasons for midwestern *C. picta*, which was correlated with an increase in estradiol concentrations, decreased testosterone concentrations, and higher estradiol:testosterone (E:T) ratios than for clutches produced earlier in the season. Clutches with higher E:T ratios were more likely to produce females, suggesting a direct effect of yolk hormone concentrations on offspring sex (Bowden et al. 2000). These potential maternal effects on sex ratios in reptiles with TSD have implications for evaluating the evolutionary potential for sex ratios, as well as comparing geographic variation in pivotal temperatures (the constant incubation temperature producing a 1:1 sex ratio). For example, if among-family variation in sex ratios has a large maternal contribution, the assumption for a fairly rapid evolutionary response of offspring sex ratios to climate change scenarios (Rhen and Lang, 1998) may be questionable. Furthermore, if pivotal temperatures change seasonally within populations, they may complicate interpretations of studies that have directly compared pivotal temperatures among populations (Bull et al., 1982b; Girondot, 1999; Mrosovsky, 1988; Vogt and Flores-Villela, 1992; Ewert et al., 1994).

Although details concerning the proximate mechanisms underlying sexual differentiation in reptiles with TSD remain unclear (Pieau et al., 1999), correlational evidence points to a key role of estrogen in initiating ovarian development (Gutzke and Bull, 1986; Crews et al., 1989), whereas testosterone appears to have a much lesser role in sexual differentiation (Crews et al., 1989; reviewed in Conley et al., 1997). Synthesis of

endogenous estradiol from androgens is thought to occur through thermally-dependent expression of the enzyme aromatase (Desvages and Pieau, 1992). Although their potential roles have mainly been investigated through the application of exogenous steroid hormones to eggs during the temperature sensitive period for sexual differentiation, recent evidence demonstrates that these hormones are already present as substrate in yolks of freshly-laid eggs in alligators and turtles with TSD (Conley et al., 1997; Janzen et al., 1998; Bowden et al., 2000), indicating an important maternal contribution to sex determination. These recent findings lead to the question of whether among-population differences in yolk steroid hormone concentrations are associated with differences in pivotal temperatures among populations.

The purpose of this study was to (1) estimate pivotal temperatures for two populations of western painted turtles (*Chrysemys picta bellii*) located in New Mexico and Illinois, (2) evaluate the reliability of the estimates during a subsequent study, and (3) to evaluate among-population differences in yolk steroid hormone concentrations as a possible explanation for differences in pivotal temperatures detected between the populations. In the first experiment, pivotal temperatures for both populations were estimated, and then eggs were incubated from each population at their respective calculated pivotal temperatures. In the second experiment, yolk estradiol and testosterone concentrations were quantified for both populations and compared to sex ratios from their respective clutches.

## Materials and methods

*Study populations*

The western painted turtle, *Chrysemys p. bellii*, exhibits TSD and has the widest latitudinal range among the four subspecies of painted turtles (Conant and Collins, 1998; Ernst et al., 1994). *Chrysemys p. bellii* are generally contiguous across their range from Texas to Manitoba, except for a disjunct population located in New Mexico (Conant and Collins, 1998). In southern New Mexico, *C. p. bellii* are generally restricted to the Rio Grande and Pecos River basins (Degenhardt et al., 1996). In this region, where climatic differences are maximized between resident populations and the rest of the subspecies' range, painted turtles are relatively rare (Stebbins, 1985). Painted turtles in New Mexico are typically located and protected in wildlife refuges, or found in much smaller numbers in remote areas that are difficult to access (32 nests in one nesting season, pers. obs.). A well-studied population of *Chrysemys p. bellii* in Illinois, however, is quite large and many clutches of eggs are relatively easy to collect, up to 200 nests in a single nesting area (Janzen and Morjan, 2001). Therefore, it was necessary to compare relatively smaller sample sizes from New Mexico *C. picta* with larger sample sizes from their conspecifics in Illinois. Gravid *C. picta* are found between late May and early July in New Mexico (Christiansen and Moll, 1973; Morjan, 2002; J. N. Stuart, pers. comm.), similar to Illinois *C. picta* (Morjan, 2002; F. J. Janzen, unpubl. data), and nesting began on nearly identical days when both populations were monitored during 1999 and 2000 (C. Morjan, 2002; F. J. Janzen, unpubl. data).

*Collection and incubation of eggs*

Four gravid female turtles from New Mexico were captured in hoop traps, two on 6 June and two on 11 July 1998 in the Rio Grande near San Marcial, Socorro County. These turtles were shipped to Iowa State University where they were injected subcutaneosly in the upper hind leg with 0.5 mL of 20u/mL oxytocin solution to induce oviposition. Eggs were collected by placing females in individual buckets containing lukewarm water 15 cm deep over 3 X 3 cm poultry wire. Turtles were subsequently released at their original location of capture.

Chrysemys picta bellii eggs were collected from 12 fresh nests between 11 June and 20 June, 1998, near the Thomson Causeway Recreation Area, Carroll County, and near Fulton Lock and Dam #13, Whiteside County, Illinois. Eggs were assigned randomly to five incubators set at the following temperatures: 27.5, 28.0, 28.5, 29.0, and 29.5°C. Within each incubator, eggs were assigned randomly to plastic shoeboxes (20 X 63 X 10 cm), and randomly to positions in a 4 X 5 matrix within shoeboxes. Each incubator contained an additional shoebox with a HOBO® XT temperature logger. Eggs within shoeboxes were half-buried in moist vermiculite (338g water for 300 g vermiculite, -150 kPa). To increase power of analysis, the smaller number of eggs from New Mexico during 1998 were assigned randomly to the three incubators set at 27.5, 28.5, and 29.5°C. New Mexico eggs were assigned randomly to the same plastic shoeboxes as the Illinois eggs within each of these three incubators.

Eggs were collected from 12 females trapped in hoop nets between 26 May and 3 June 2000 near San Marcial, Socorro County, New Mexico, and on the Bosque del Apache National Wildlife Refuge, Socorro County, and from three freshly laid, natural nests found

on the Refuge. Females were induced to oviposit at the field site as described above and released shortly thereafter. Two eggs were chosen randomly from each clutch and frozen immediately. The remaining eggs from the four females collected outside of the refuge were taken to Iowa State University on 9 June and incubated under conditions described above at their expected pivotal temperature of 28.4°C (see Results). In Illinois, clutches from the Thomson Causeway Recreation Area were collected from nine freshly laid, natural nests and from 11 gravid females captured by hoop nets between 23 May and 1 June 2000. Females were induced to oviposit, as described above, at the site of their collection and released when eggs were laid. From each of these clutches, two eggs were chosen at random and frozen immediately. The remaining eggs were packed in moist sand in coolers and transported to Iowa State University within a few days of collection, and incubated as described above at their expected pivotal temperature of 27.7°C (see Results).

For both years, boxes within incubators were rehydrated weekly and rotated daily, both vertically and horizontally, to account for possible temperature gradients within incubators. Hatchlings were kept for at least a month to allow for yolk reabsorption before euthanization by injection of 0.6 ml of a 1:1 solution of water and Sleepaway (Janzen et al., 1998) or by freezing (Packard et al., 2002). To eliminate observer bias, hatchling identification was coded so that clutch of origin and incubation temperature were unknown during the sexing process. Sexes were determined by macroscopic inspection of the gonads under a dissecting microscope (Janzen, 1994). Specimens were preserved in ethanol and are stored at Iowa State University.

Incubation temperatures were determined by calculating the mean of temperatures taken every ca. 15 min for at least one week in the incubators. From these measurements,

actual temperatures during 1998 were estimated as 27.2, 27.7, 28.3, 28.4, and 29.9°C for the incubators set at 27.5, 28.0, 28.5, 29.0, and 29.5°C, respectively. Standard deviations in temperatures were less than 0.2°C for all incubators except for the incubator set for 28.0°C, which was less than 0.3°C. During 2000, actual temperatures from the 27.7°C incubator were estimated as 27.5 ± 0.2°C, and actual temperature from the 28.4°C incubator was estimated as 28.3 ± 0.2°C.

*Radioimmunoassay*

For analysis of testosterone and estradiol-17β concentrations within yolks of eggs collected during 2000 (two eggs from each of 15 New Mexico clutches and two from each of 20 Illinois clutches), a cross-section of the center of the frozen yolk was taken from each egg, placed in an Eppendorf tube, and homogenized when thawed. To minimize potential effects of variation in water content in the analyses, a subsample from each of the homogenized yolks was dried in a lyophilizer, and approximately 30 mg (weighed to the nearest 0.01 g) of the dry mass was combined with 400 µl of distilled water with three glass beads. The dried yolk was resuspended using a sonicator water bath. To calculate recoveries through the extraction process, 2000 CPM [$^3$H]testosterone and 2000 CPM [$^3$H]estradiol-17β was added to each sample.

Samples were extracted in ether and run through chromatography columns following the method described in Reed and Vleck (2001). After adding extracted samples to columns, I ran four consecutive solutions through the columns: 4 ml of 100% iso-octane, 4 ml of 2% ethyl acetate in iso-octane, 5 ml of 20% ethyl acetate in iso-octane, and 4.5 ml of

40% ethyl acetate in iso-octane. Testosterone was collected from the 20% ethyl acetate in iso-octane phase, and estradiol was collected from the 40% ethyl acetate in iso-octane. A subsample was collected from each of these eluates to assess recoveries of testerone and estradiol.

Testosterone and estradiol-17β concentrations were measured using kits of competitive binding of iodonated hormones with specific antibodies (Diagnostic Systems Laboratories, Inc., Webster, Texas), as described by Reed and Vleck (2001). Samples were run in triplicate and compared to a standard curve that ranged from 0.025 to 24 ng/ml for testosterone and from 0.78 to 750 pg/ml for estradiol (Figure 1). Recovery values ranged from 29 - 60% with an average of 44% for testosterone, and from 25 - 69 % with an average of 50% for estradiol. All samples were run in the same assay. Intra-assay coefficients of variation, based on triplicate samples, averaged 15% for testosterone and 14% for estradiol.

*Statistical analyses*

Hormone concentrations were first adjusted for recovery and log-transformed to normalize the data. Statistical analyses were performed in JMP 3.2.1 for Macintosh (SAS Institute, 1993). Data for the 1998 incubation experiments to assess population differences in pivotal temperatures were first analyzed using nominal logistic regression in JMP. An analysis using likelihood ratio tests was conducted using incubation temperature (as categorical data since separate incubators were associated with each temperature regime), population, their interaction, and clutch nested within population as effects and sex as a response. Pivotal temperatures were then calculated using maximum likelihood (Mac

version 1.3, Girondot, 1999). The model used in this method describes the function in which the frequency of males (*sr*) is produced at a constant temperature, t:

$$sr(t) = \frac{1}{1 + e^{\left(\frac{1}{S}(P-t)\right)}},$$

where $P$ is the pivotal temperature and $S$ describes the shape of the transition in sex ratios across temperatures. Further details are described in Girondot (1999).

Additional analyses were conducted on the 1998 data from Illinois to evaluate potential seasonal differences in sex determination. The 12 Illinois clutches were grouped into two time categories likely to represent primary clutches and those representing second clutches from females within the nesting season. Laying date was separated into the two time categories rather than continuous data because statistical analyses do not permit evaluating the effects of clutches nested within a continuous variable (laying date). However, since collections occurred over two short and disjunct time periods, results are unlikely to be affected by this choice. Thus the nine clutches collected between 11 and 13 June constituted the early season clutches, whereas the remaining three collected between 18 and 20 June constituted the late season clutches. These groupings were likely to naturally correspond with early versus late clutches since nesting began in late May, females typically lay two clutches per season, and inter-clutch nesting intervals span about two weeks in this population (F. J. Janzen, unpubl. data; R. Bowden, pers. comm.). Effects of time category, clutch within each time category, and incubation temperature on offspring sex were evaluated using nominal logistic regression. The four clutches from New Mexico were too few to allow a similar analysis to be conducted for that population.

## Results

For the 1998 incubation experiment, incubation temperature, clutch within population, and population significantly affected offspring sex (Table 1). The maximum-likelihood method (Girondot, 1999) also detected significant differences in sex determination between the populations ($P < 0.05$), and calculated the pivotal temperature for the New Mexico population as 28.40 (95% CI: 27.81 - 28.99), with a slightly lower pivotal temperature for the Illinois population at 27.72 (95% CI: 27.53 - 27.91).

When eggs from New Mexico and Illinois were incubated near their estimated pivotal temperatures during 2000, only 3 eggs out of 22 (13.6%) from New Mexico and 5 eggs out of 140 (3.6%) from Illinois produced females. By using temperature as continuous data rather than blocked by incubator from the 1998 experiments, I used inverse prediction to construct 95% confidence intervals for the incubation temperatures expected to produce a 13.6% or 3.6% sex ratio from New Mexico and Illinois, respectively, during 1998. These intervals are 24.0 - 27.8°C for New Mexico, and 25.2 - 26.7°C for Illinois, temperatures well below those used to achieve such male-biased sex ratios for both populations during 2000 (28.3°C and 27.5°C, respectively). Although using temperature as continuous rather than categorical data violates the assumption of independent predictor variables, this comparison is likely robust because inverse prediction from this method provides identical pivotal temperatures and similar confidence intervals, albeit slightly wider (New Mexico: PT = 28.40, CI = 27.61 - 29.38; Illinois: PT = 27.72, CI = 27.46 - 27.91), compared to those provided by Girondot's (1999) maximum likelihood method.

Significant differences in sex determination between early versus late clutches within the Illinois incubation experiment during 1998 were detected using logistic regression

(Table 2). The pivotal temperature calculated for the early clutches using maximum likelihood (Girondot, 1999) was 27.84°C (CI: 27.58 - 28.10), whereas the pivotal temperature for the late clutches was lower at 27.49°C (CI: 27.23 - 27.75). Similarly, maximum likelihood (Girondot, 1999) demonstrated significant differences in the distributions of sex ratio between early and late clutches ($P < 0.05$). These results indicate that later clutches were more likely to produce females than earlier clutches in Illinois.

Figures 2 and 3 display concentrations of testosterone, estradiol, and E:T ratios for eggs within clutches from New Mexico and Illinois, respectively, from early season clutches during 2000. Note that in Figure 2 the scales on the Y-axis for estradiol and E:T ratios are an order of magnitude larger than those displayed from Illinois in Figure 3. Significant variation in concentrations of estradiol and E:T ratios were found among clutches within both populations, and between populations (Table 3). However, no differences in testosterone concentrations were found either within or between populations (Table 3). Estradiol concentrations were highly correlated between eggs within clutches for both New Mexico ($r = 0.94$, $P < 0.0001$) and Illinois ($r = 0.67$, $P = 0.002$), but testosterone concentrations were not correlated between eggs within clutches for either population ($P > 0.40$ for both).

Eggs from clutches producing females did not exhibit higher or lower steroid hormone concentrations or E:T ratios ($P > 0.3$ for all) than those from clutches producing all males (Figures 2 and 3). Although eggs from clutch 14 from Illinois had the highest E:T ratio and also produced females, the other clutches from that population producing females (8 and 18) exhibited some of the lowest E:T ratios in the population (Figure 3). Although not statistically significant, mean concentrations of yolk testosterone decreased ($r = -0.42$, $P$

= 0.16), mean concentrations of yolk estradiol increased ($r = 0.39$, $P = 0.15$), and E:T ratios increased ($r = 0.47$, $P = 0.08$) during the 9-day collection period in New Mexico, showing similar directional tendencies with seasonal trends reported for *C. picta* in Indiana by Bowden et al. (2000). Similarly, mean testosterone concentrations decreased for clutches collected through the 10-day period in Illinois, but this result was not statistically significant ($r = -.40$, $P = 0.08$). No temporal changes occurred for mean estradiol concentrations or for E:T ratios in Illinois ($P > 0.50$ for both). No differences in mean hormone concentrations were found between clutches collected from natural nests and those collected from females injected with oxytocin, after accounting for population differences (ANCOVA: testosterone: $F(1,34) = 0.85$, $P = 0.37$; estradiol: $F(1,34) = 0.79$, $P = 0.38$; all interactions n.s.).

## Discussion

Among-population geographic variation in pivotal temperatures generally does not vary in expected patterns with latitude (high latititudes with lower pivotal temperatures) in turtles with TSD (Bull 1982b; Mrosovsky, 1988; Vogt and Flores-Villela, 1992; Ewert et al. 1994). In contrast, New Mexico eggs exhibited a higher pivotal temperature than eggs from Illinois in 1998, in accordance with the expectation that higher pivotal temperatures should be found in hotter climates. Although statistically significant, this difference in pivotal temperatures was small (0.7°C) and its biological significance on sex ratios in natural populations is unknown. Given that air temperatures during the incubation season average 2°C higher at the field site in New Mexico than in Illinois (National Oceanic and Atmospheric Administration, 2001; Janzen, 1994a), other factors may also need to be

considered when evaluating the maintenance of sex ratios in these populations. First, although sex ratios of adults do not appear to be female-biased in New Mexico (Morjan, 2002) and offspring sex ratios are expected to be 1:1 at the field site in Illinois (Janzen, 1994a), the possibility that *C. picta* primary sex ratios are skewed in New Mexico has not been ruled out. Secondly, although females typically laid eggs at similar times between the populations (generally late May through early July), a few females in New Mexico produced some clutches during mid-July in New Mexico during 1998 (see Methods). How frequently these very late clutches occur in New Mexico, and the nest temperatures they are expected to experience, are unknown. A concurrent study also shows that nests from both populations tend to experience similar thermal conditions (Morjan, 2002), suggesting that nest-site choice by females may be an important component in the maintenance of sex ratios between the populations. Conclusions concerning how sex ratios may be maintained in *C. picta* will need to account for many of these aspects of the organism's life history.

Early versus late seasonal effects (Bowden et al., 2000) or changes in incubation temperature over time are unlikely to have influenced the differences in pivotal temperatures detected during 1998, because the clutches that were collected from New Mexico bracketed the time that eggs were collected from fresh nests in Illinois that year. However, eggs from both populations incubated at their respective calculated pivotal temperatures for the overall 1998 season produced extremely male-biased clutches during 2000. This result may be due to the fact that although eggs from both populations were collected simultaneously during 2000, they were collected only at the start of the nesting season. For example, Bowden et al. (2000) reported a seasonal decrease in sex ratio (proportion of males) from *C. picta* eggs incubated at pivotal temperature. Similarly, in this study, sex ratios tended to be male-

biased early in the season and female-biased later in the season for eggs incubated across several temperatures in Illinois. This result supports a seasonal decrease in sex ratio in an additional population of *C. picta*, and that this effect occurs at temperatures throughout the transition range of sex determination in addition to pivotal temperatures.

Bowden et al. (2000) reported significant correlations between both estradiol and E:T ratios with sex ratios among clutches of *C. picta*, such that higher estradiol concentrations and E:T ratios produced more females. Although such a trend was not found in this study, it is not surprising given the few females produced at each population's expected pivotal temperature. If such correlations of endogenous steroid hormone concentrations with sex ratios can be generalized to explain among-population differences in pivotal temperatures, higher concentrations of estradiol or higher E:T ratios might be expected in populations producing more females (exhibiting a lower pivotal temperature). In contrast, although eggs from Illinois exhibited a lower pivotal temperature than those from New Mexico, estrogen levels and E:T ratios were markedly lower in clutches from Illinois, opposite to the expected direction. These results suggest that the correlations between yolk steroid hormone concentrations and sex ratios within populations as reported by Bowden et al. (2000) may not be a general mechanism for explaining differences in sex determination among populations. However, since Bowden et. al (2000) detected little correlation between these factors when looking at early season clutches only (Bowden et al., 2000; 2001), and this study assayed hormone levels for early season clutches, further studies addressing patterns in sex determination over extended collecting periods for the populations are necessary before making conclusions on its generality.

Yolk estradiol concentrations were markedly higher in *C. picta* eggs from New Mexico than their Illinois conspecifics. In birds, crocodilians, and turtles, yolk steroid hormone concentrations are correlated with maternal circulating hormone levels (Schwabl, 1996; Conley et al., 1997; Lipar et al., 1999; Bowden et al., 2001; Janzen et al., 2002). Circulating levels of androgens and estrogens are also correlated with photoperiod in some birds (Schwabl, 1996; Mishra and Tewary, 1999). Further research could investigate the role of photoperiod in maternal hormone profiles in reptiles, and its potential role in latitudinal variation in yolk hormone concentrations.

Although clutches varied markedly (as high as an order of magnitude) in estradiol concentrations, no differences were detected across clutches or populations for testosterone concentrations with the sample size used. Although the testosterone levels from two serially diluted *C. picta* yolks did not closely follow the standard curve (Figure 1A), the levels in these eggs were initially low even at their highest concentration. Most testosterone concentrations were well within the detectable levels of the assay (0.2 – 3 ng/g; compare to Figure 1A). Similar to Bowden et al. (2000), estradiol concentrations from eggs within clutches were highly correlated with those of their siblings, but no such correlation was found for testosterone concentrations. However, Bowden et al. (2000) found significant differences among clutches for yolk testosterone concentrations in addition to estradiol for *C. picta*, with a larger sample size than used in this study. Conley et al. (1997) reported significant among-family variation in yolk estradiol concentrations prior to gonadal differentiation in American alligators (*Alligator mississippiensis*), and high concentrations (but no significant among-clutch differences) in testosterone or androstenedione, two important substrates for estradiol synthesis. In contrast, Janzen et al. (1998) found among-

clutch variation in testosterone rather than in estradiol in *Trachemys scripta*, *Chelydra serpentina*, and *Chrysemys picta*. Egg yolks from TSD species also exhibited markedly higher concentrations of testosterone than those from non-TSD species, and higher testosterone concentrations were also weakly associated with male-biased sex ratios for clutches of common snapping turtles (*Chelydra serpentina*) incubated at pivotal temperatures (Janzen et al., 1998). Janzen et al. (1998) reported much higher yolk testosterone concentrations for *C. picta* (75 – 250 ng/g) than those reported by Bowden et al. (2001) (0.2 – 1.2 ng/g) and in this study (0.2 – 3 ng/g). These differences are likely due to differing assay methods, particularly since this study used the same *C. picta* population used by Janzen et al. (1998).

Most turtles exhibit Pattern I TSD, such that warmer incubation temperatures produce females, and cooler incubation temperatures produce males (Ewert and Nelson, 1994). If a seasonal trend in sex determination is a general pattern for turtles with Pattern I TSD, such that more males are produced early in the season, the "pivotal temperature" will be higher for earlier clutches than for later clutches. Indeed, this shift in pivotal temperature occurred during the 1998 incubation season in Illinois, and sex ratios from clutches collected early in the subsequent season indicate a higher temperature was required to produce a 1:1 sex ratio. However, this trend will need to be evaluated in other TSD taxa before general conclusions can be drawn. Fortunately, the possibility of a shifting pivotal temperature does not appear to have confounded analyses of geographic variation in sex determination in North American turtles with TSD reported by Bull et al. (1982a) and Mrosovsky (1988). Although eggs were collected earlier in the season for the southernmost populations in both studies, no cline in pivotal temperatures was reported by Mrosovsky (1988), and a cline in

the opposite direction was reported by Bull et al. (1982), such that earlier clutches from southern populations exhibited a lower pivotal (i.e., "threshold") temperature than clutches collected later from northern populations. It is unclear when eggs from various populations of *C. picta* were collected for geographic comparisons of sex determination reported by Ewert et al. (1994) or for *T. scripta* and *C. serpentina* by Vogt and Flores-Villela (1992). However, the patterns of sex determination reported in these two studies also do not suggest such a confounding seasonal effect, since pivotal temperatures were again reported to be higher in more northern localities. Given the recent progress in statistical methods allowing evaluation of geographic variation in sex determination (Girondot, 1999), more attention to additional sources of variation affecting sex determination is necessary to ensure that reported differences among populations are not artifactual.

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**Figure 1.** Sample standard curves from **(A)** a yolk testosterone radioimmunoassay, and **(B)** a yolk estradiol radioimmunoassay. The first panel **(A)** shows the relationship between concentration of testosterone (ng/ml) and percentage binding of radiolabeled testosterone to testosterone antiserum, and two serially diluted yolk samples from two *Chrysemys picta* eggs. The second panel **(B)** shows the relationship between concentration of estradiol (pg/ml) and percentage binding of radiolabeled estradiol to estradiol antiserum, and two serially diluted yolk samples from two *Chrysemys picta* eggs.

**Figure 2.** Concentrations of **(A)** testosterone, **(B)** estradiol, and **(C)** estradiol:testosterone ratios in dried yolks of painted turtle eggs from New Mexico collected at the beginning of the nesting season. Remaining eggs from clutches 3, 4, 5, and 7 were incubated in the laboratory at 28.3°C; clutches 3 and 4 produced some female hatchlings (marked with an X) while those from clutches 5 and 7 produced all male hatchlings. Means (± SD) for the combined data are provided in each panel.

**Figure 3.** Concentrations of **(A)** testosterone, **(B)** estradiol, and **(C)** estradiol:testosterone ratios in dried yolks of painted turtles from Illinois at the beginning of the nesting season of 2000. Eggs marked with X are from clutches that produced some female hatchlings, while all remaining clutches produced all male hatchlings when incubated in the laboratory at 27.5°C. Means (± SD) for the combined data are provided in each panel.

**Table 1.** Effects of incubation temperature, population, and clutch nested within population on sex of *Chrysemys picta* eggs incubated at constant temperatures. Likelihood ratio values (LR $\chi^2$) are derived from logistic regression with an individual's sex as the response variable. Illinois eggs (n = 140) were incubated at 5 temperatures and New Mexico eggs (n = 32) at 3 of these temperatures.

| Effect | df | LR$\chi^2$ | P |
|---|---|---|---|
| Temperature | 4 | 98.80 | <0.0001 |
| Population | 1 | 8.92 | 0.0028 |
| Temperature X Population | 2 | 0.21 | 0.90 |
| Clutch | 14 | 31.46 | 0.0048 |

**Table 2.** Effects of incubation temperature, time category (early versus late clutches) and clutch nested within time category on sex determination for 12 clutches of *Chrysemys picta bellii* eggs (n = 140) collected from Thomson, Illinois, during 1998. Likelihood ratio values (LR $\chi^2$) are derived from logistic regression with an individual's sex as the response variable.

| Effect | df | LR$\chi^2$ | P |
|---|---|---|---|
| Temperature | 4 | 79.80 | <0.0001 |
| Time category | 1 | 7.81 | 0.0052 |
| Clutch | 10 | 22.80 | 0.012 |

**Table 3.** Effects of population and clutch nested within population on concentrations of testosterone, estradiol, and estradiol:testosterone ratios in yolks of *Chrysemys picta* eggs from New Mexico (n = 30) and Illinois (n = 38).

| Response | Source of variation | df | F | P |
|---|---|---|---|---|
| Testosterone | Population | 1 | 1.31 | 0.26 |
| | Clutch | 33 | 1.56 | 0.098 |
| Estradiol | Population | 1 | 14.40 | 0.0006 |
| | Clutch | 33 | 8.23 | < 0.0001 |
| Estradiol:Testosterone | Population | 1 | 9.07 | 0.005 |
| | Clutch | 33 | 7.44 | < 0.0001 |

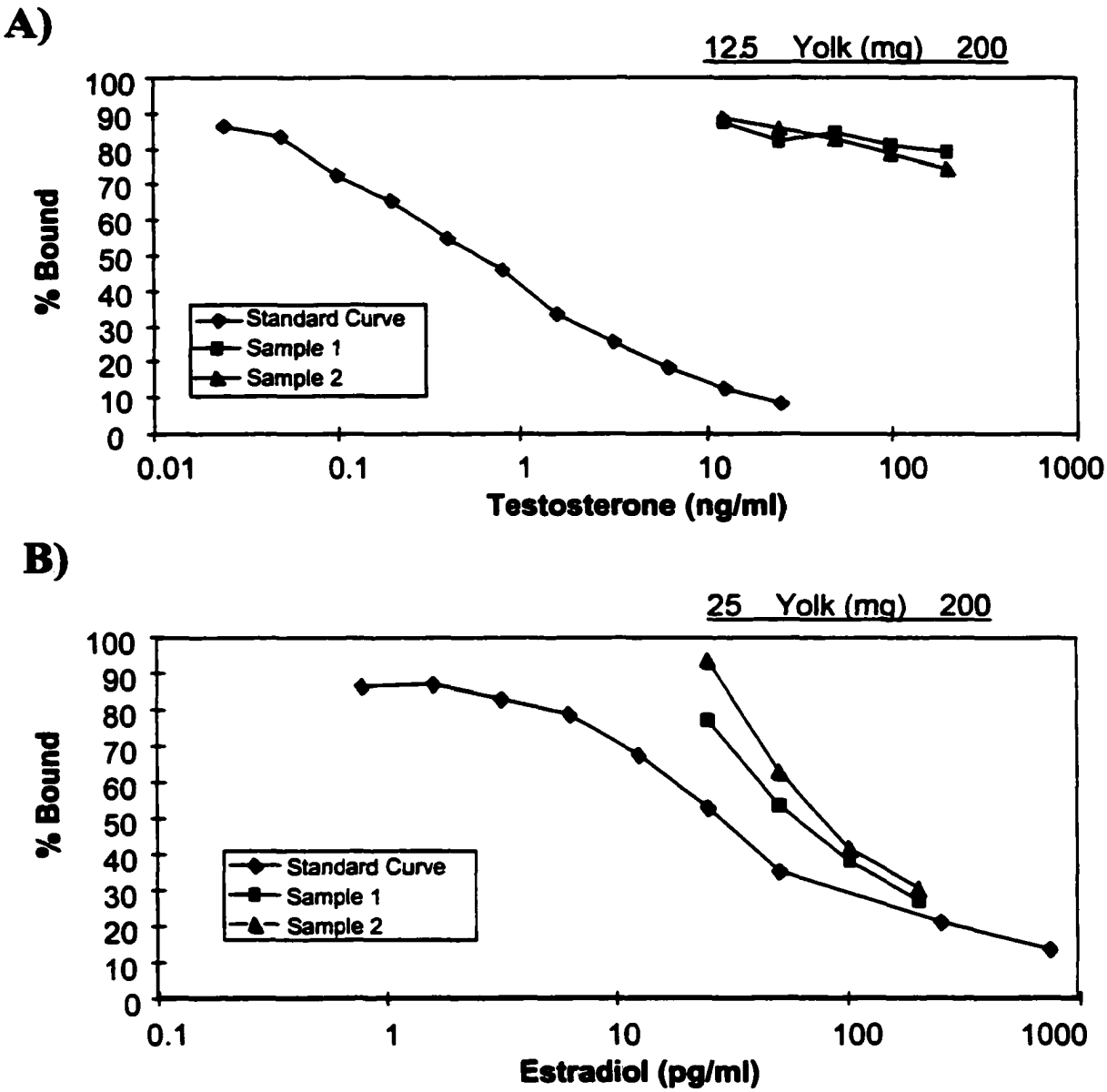
A)
125 Yolk (mg) 200
% Bound
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
Testosterone (ng/ml)
Standard Curve
Sample 1
Sample 2
B)
25 Yolk (mg) 200
% Bound
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
Estradiol (pg/ml)
Standard Curve
Sample 1
Sample 2

**Figure 1.**

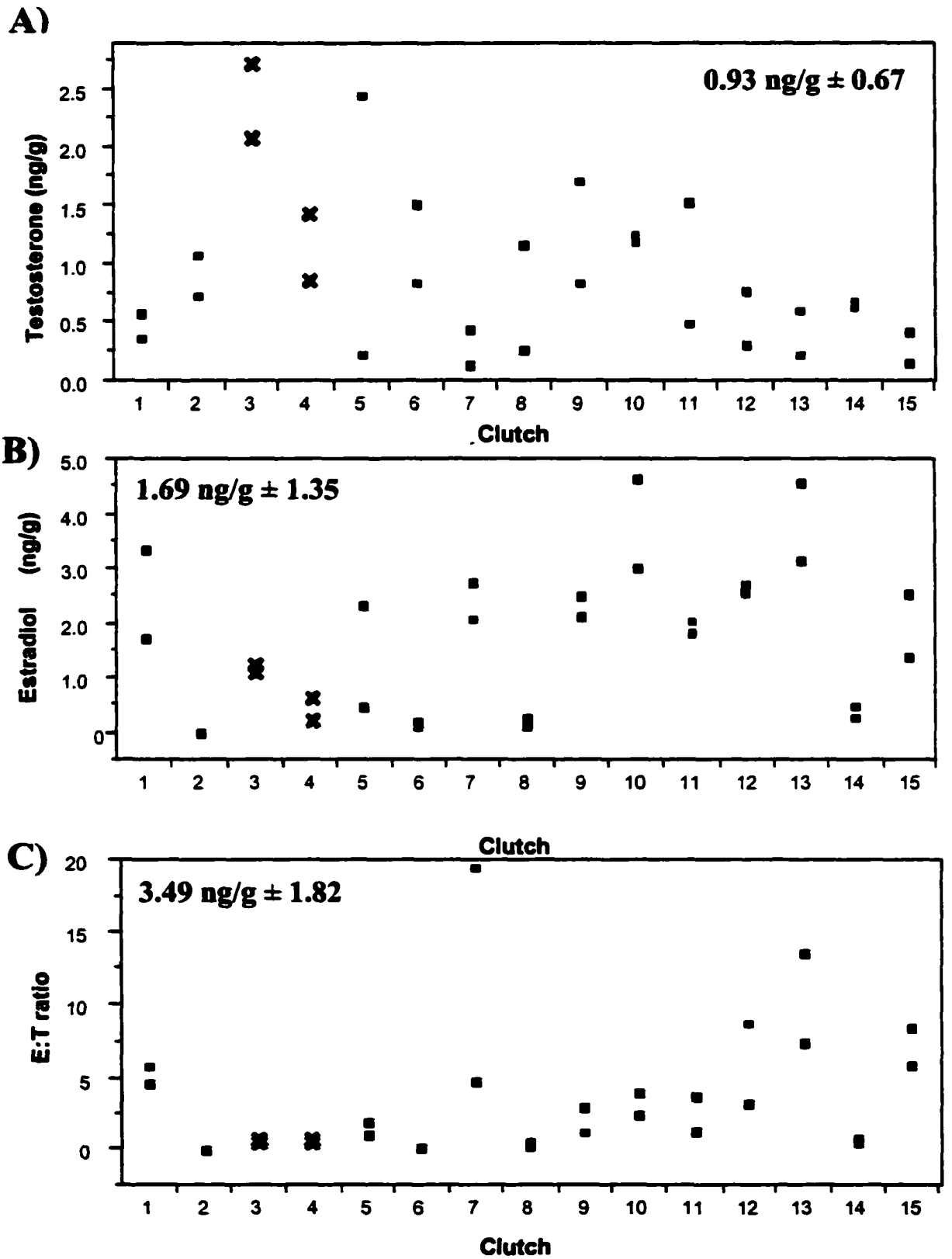
A)
0.93 ng/g ± 0.67
Testosterone (ng/g)
0.0
0.5
1.0
1.5
2.0
2.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Clutch
B)
1.69 ng/g ± 1.35
Estradiol (ng/g)
0
1.0
2.0
3.0
4.0
5.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Clutch
C)
3.49 ng/g ± 1.82
E:T ratio
0
5
10
15
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Clutch

**Figure 2.**

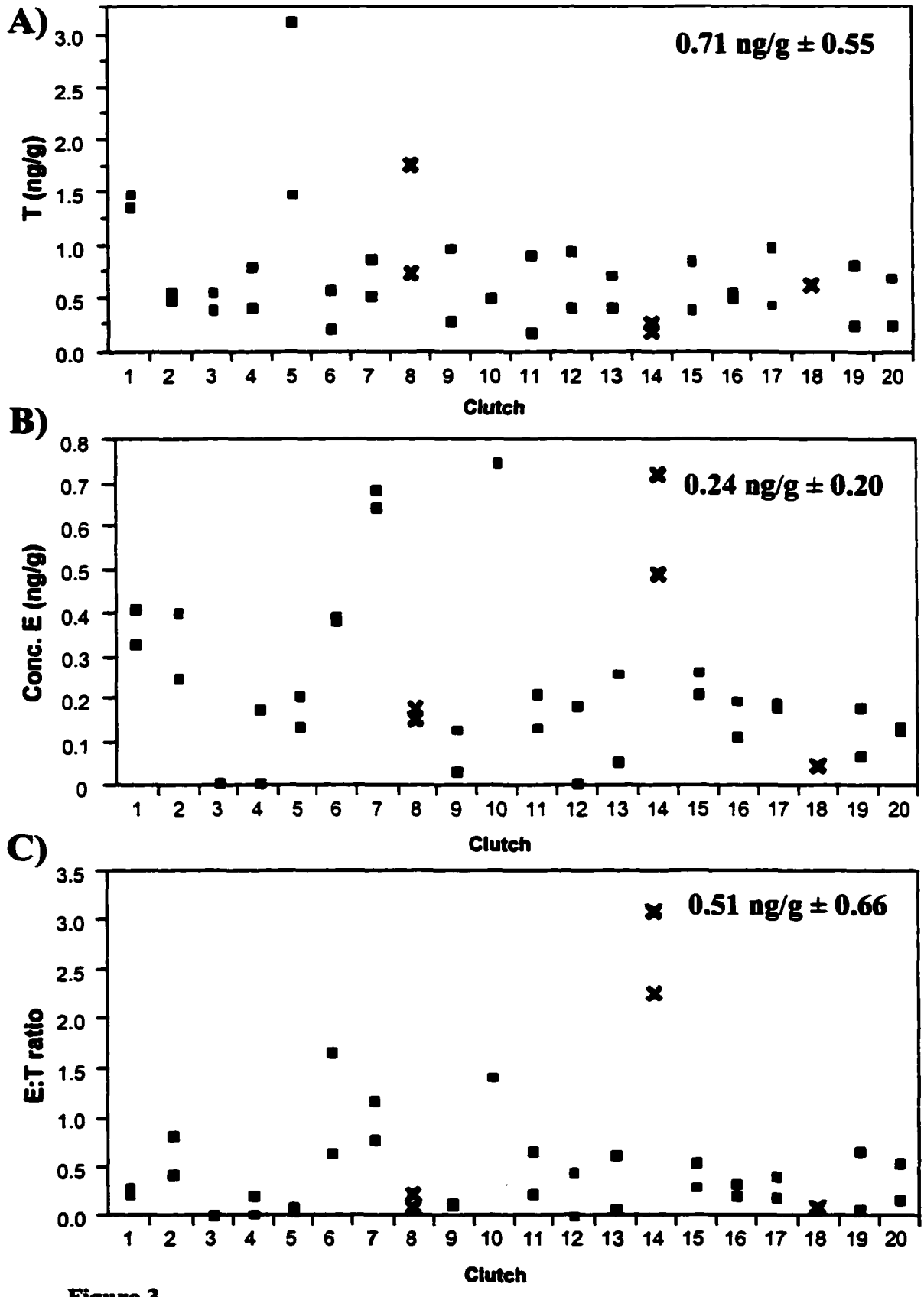
A)
0.71 ng/g ± 0.55
T (ng/g)
Clutch
B)
0.24 ng/g ± 0.20
Conc. E (ng/g)
Clutch
C)
0.51 ng/g ± 0.66
E:T ratio
Clutch

**Figure 3.**

## CHAPTER 4.

**Variation in nesting patterns and effects on nest temperatures in two populations of painted turtles (*Chrysemys picta*) with temperature-dependent sex determination.**

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Carrie L. Morjan

Department of Zoology and Genetics, Iowa State University

Ames, IA 50011

## ABSTRACT

Mechanisms maintaining sex ratios in populations with temperature-dependent sex determination (TSD) remain elusive. Theoretical models suggest that primary sex ratios may evolve through genetic variation and selection on two traits in this system: sex ratio of offspring in response to thermal environment, and maternal behavior affecting thermal incubation conditions. Although geographic variation in the former trait has been widely investigated in reptiles exhibiting TSD, no previous studies have directly addressed geographic variation in the latter trait. I evaluated patterns of nest-site selection and its effects on thermal and hydric nest conditions for a population of painted turtles (*Chrysemys picta bellii*) exhibiting TSD in New Mexico. These results are compared to data collected from a well-studied, conspecific population experiencing relatively cooler climatic conditions in Illinois. Females from both populations nested at similar times and under relatively less canopy vegetation cover than available to them; however, New Mexico females differed by placing nests extremely close to standing water. Although vegetation cover significantly lowered nest temperatures in New Mexico, so did increased soil moisture

around nests, which was negatively correlated with distance from nests to water. Nests from both populations experienced similar mean July temperatures, despite New Mexico experiencing hotter climatic conditions than typical during the field seasons. Since females in New Mexico nested in habitats most closely associated with maximizing moisture around nests rather than to directly reduce nest temperatures, nesting patterns may primarily reflect selection for microhabitats affecting offspring survivorship or traits other than sex.

**KEYWORDS**: nest-site choice, nesting behavior, maternal effects

## Introduction

Maternal effects may provide a powerful mechanism for local adaptation and divergence among populations. Oviposition behavior by females is especially important in this context, because, coupled with progeny response to oviposition environment, relatively rapid adaptation to local conditions may occur (Wade 1998). Since incubation temperatures affect offspring sex in reptiles with temperature-dependent sex determination (TSD), females may potentially influence the sex of their offspring through preferences for specific thermal oviposition environments. Although many reptiles with TSD exhibit wide geographic ranges (Janzen and Paukstis 1991, Ernst et al. 1994), the role of maternal nesting behavior in mitigating differing climatic conditions on nest temperatures across these ranges has largely remained uninvestigated. Without such compensating mechanisms for climatic differences, skewed sex ratios might then occur across their distributional ranges.

In addition to female nesting behavior affecting nest thermal qualities, variation in and selection on thermal response of offspring sex may also be important for sex ratios to

evolve in this system (Bull 1983, Bulmer and Bull 1982, Karlin and Lessard 1986). Consequently, understanding geographic variation in both of these traits may provide important insight into mechanisms of sex ratio evolution, the distribution of extant TSD taxa, and how these populations may respond to climatic changes. For example, local and geographic variation in thermal response of offspring sex ratio has been studied extensively in sea turtles and North American freshwater turtles (Bull et al. 1982a, 1982b, Mrosovsky 1988, Ewert et al. 1994, Rhen and Lang 1998, Bowden et al. 2000). If thermal response of offspring sex can evolve to maintain sex ratios across varying thermal regimes, pivotal temperatures (the constant temperature producing a 1:1 sex ratio in the laboratory) might exhibit a cline concordant with latitude. However, pivotal temperatures have generally varied by only 1 - 2 °C within most taxa, and in the cases where latitudinal clines were found at all, they generally occur in patterns opposite to the expected direction (Bull et al. 1982b, Mrosovsky, 1988, Vogt and Flores-Villela 1992, Ewert et al. 1994, Mrosovsky 1994, Ackerman 1997). Instead, variation in nesting behavior by females affecting nest temperatures has been thought to be more likely to maintain sex ratios for TSD taxa (Bull et al. 1982a, 1982b, Bulmer and Bull 1982).

I evaluated geographic variation in nesting patterns using a previously unstudied population of painted turtles (*Chrysemys picta*) inhabiting relatively extreme climatic conditions in New Mexico. I present data from *C. picta* located on the Mississippi River in Illinois for comparison. Painted turtles are widely distributed, well-studied North American freshwater turtles exhibiting Pattern I TSD (Ewert et al. 1994), such that warmer incubation temperatures produce females and cooler incubation temperatures produce males. In particular, the western painted turtle (*C. picta bellii*) has the widest latitudinal distribution

among the four subspecies that have been described, ranging from Manitoba to Texas (Conant and Collins 1998). Female *C. p. bellii* from both populations were expected to nest at similar times during this study because timing of reproduction was found to be fairly similar between *C. p. bellii* from New Mexico and Wisconsin (Christiansen and Moll 1973). Mean July air temperatures at the southern range of this subspecies along the Rio Grande in New Mexico, including the selected site at the Bosque del Apache Refuge, average greater than or equal to 25.5°C (Wood 1996). Such climatic conditions (mean July air temperatures) during the period of egg incubation strongly affect annual cohort sex ratios within the Illinois population (Janzen 1994a, F. J. Janzen, unpubl. data), and this temperature is expected on average to produce 100% female offspring (Janzen, 1994a) and cause extinction in the Illinois population (Morjan, 2002). Pivotal temperatures also exhibit little variation between the New Mexico and Illinois populations (0.7°C), unlikely to be the sole mechanism responsible for maintaining sex ratios across the climatic differences (Morjan, 2002).

Vegetation surrounding turtle nests affects nest temperatures, and may be an important cue for nest-site selection (Vogt and Bull 1984, Roosenburg 1996, Wilson 1998, Weisrock and Janzen 1999). In the Illinois population, canopy vegetation cover over nests, particularly cover from the south and west directions, affects relative nest temperatures each year (Weisrock and Janzen 1999, C. Morjan and F. J. Janzen, submitted) and offspring sex ratios (Janzen 1994b, Weisrock and Janzen 1999, Valenzuela and Janzen 2001) during years experiencing intermediate climatic conditions. Furthermore, females tended to nest under less canopy vegetation than available at the nesting site (Janzen and Morjan 2001). Since observations on several populations (albeit different species) of North American freshwater

turtles suggest that nests tend to be laid at more exposed areas in the north and close to or under vegetation in the south (Ewert 1976, Janzen and Morjan 2001, Wilson 1998, Vogt and Flores-Villela 1992), I focused particularly on the role of vegetation over nests between the populations. Specifically, I investigated whether female *C. picta* in New Mexico laid eggs under relatively greater vegetation cover than available to them, and generally under greater vegetation cover than females from the Illinois population. Secondly, I evaluated the possibility that females in New Mexico may dig deeper nests than those from Illinois. I also investigated the effects of two major nesting patterns that I observed on nest temperatures in New Mexico, and compared nest temperatures to those that have been collected at the Illinois population for several field seasons. Finally, I provide preliminary information on the timing of nesting in New Mexico females and on adult sex ratios. This information is a first attempt to empirically quantify geographic variation in nesting ecology that is presumed to exist for populations exhibiting TSD.

## Methods

*Nesting activity and nest measurements*

I searched for nests and nesting activity at the Bosque del Apache National Wildlife Refuge, Socorro County, New Mexico (33°46' N, 106°54' W), daily from 13 May until 9 July, 1999, and from 25 May until 13 June, 2000. Intact nests were identifiable as a heart-shaped, thick plug of mud resting on the ground's surface. This plug was easily peeled off of the nest and the eggs were visible in the cavity inside. Nests destroyed by predation were easily found because broken eggshells were scattered around the excavated cavity.

Nesting females were frequently spotted by driving around the perimeter of the major ponds of the Bosque del Apache Refuge every 1.25 hr from 1500 to 2000 h, and were observed from a vantage point using binoculars to minimize disturbance. I captured females while they completed their nests and marked them uniquely by filing their marginal scutes on the carapace. Plastron lengths were measured with a ruler, and turtles were released immediately after these observations were made.

I measured several characteristics of nests in New Mexico, including nest depth, canopy vegetation, height and percentage of surface vegetation, slope and aspect of nests, relative soil moisture, and distance from nests to water. I excavated intact nests to count eggs and measure nest depths, and reburied the eggs in the positions that they were found. I measured percentage of canopy vegetation directly over all nests, depredated and intact, by using the Model A spherical densiometer following the same methods used to quantify vegetation over *C. picta* nests in Thomson, Illinois (Janzen 1994b, Weisrock and Janzen 1999). The average percentage vegetation cover from the south and west (Janzen 1994b, Weisrock and Janzen 1999, Janzen and Morjan 2001, Valenzuela and Janzen 2001) was used for subsequent analyses. I also measured the percentage of surface vegetation surrounding all nests by centering a hoop 1-m in diameter around each nest and visually estimating the percentage of ground vegetation enclosed by the hoop, to the nearest ten percent. Vegetation height closest to each nest in each of the four directions was measured with a ruler. I measured slope of nests by placing a dial protractor on a 50 X 4 cm board centered over the nest, parallel to the direction of the steepest slope. Aspect was taken by facing down the direction of the steepest slope over the nest and measuring in degrees with a compass. During 1999, I recorded electrical conductivity of soil (a measure of relative soil

moisture) on a scale from 1 - 10 by inserting a 15-cm probe 10-cm away from the nest and parallel to the water's edge. I also attempted to use tensiometers but this technique failed, likely due to the composition and dryness of the soil. During 2000 I measured percent water content of soil at nests. This procedure involved taking a 2-cm diameter soil core at a 10-cm distance from each nest and sealing half of each core (from 0-10 cm depth and 10-20 cm depth) in separate plastic bags, weighing bags to the nearest 0.01 g at the field site, and reweighing the bags after drying the samples to a constant mass in a drying oven in the laboratory at Iowa State University. Average percent water content from the two halves were used in analyses.

Methods for data collected on *C. picta* females and nests at the Thomson Causeway, Whiteside County, Illinois (41°57' N, 90°7'W) are described in Janzen and Morjan (2001). Data from this field site used for comparative purposes, including nesting dates, canopy vegetation cover over nests, nest temperatures, and geographic locations of nests, have been collected from 1995-2000 (Weisrock and Janzen 1999, Janzen and Morjan 2001, Valenzuela and Janzen 2001, Morjan and Janzen, unpubl. data). Nest depths were also measured during 2000.

*Sampling nesting habitat*

To address differences in vegetation characteristics between nest sites and available sites in New Mexico during 1999, I sampled 40 sites around the perimeters of the pond complex where most nesting occurred. I stratified the nesting area into eight sections to correspond with the eight sides of the bodies of water from which turtles were seen emerging during the nesting season. For each of these sections, I sampled five random sites

by counting the number of paces along the section, choosing five random numbers within the number of paces, counting the paces from a starting point at the end of the section and sampling at these random pace numbers at a distance of 2.5 m from the edge of the water. I chose to take samples at 2.5 m from the water because that was close to the average distance from the water to the nests during the 1999 nesting season (see Table 1). Percent ground cover, percent canopy vegetation cover, and surface vegetation height for the sampled sites was measured by centering the 1-m hoop over the site to be sampled and proceeding as described previously. I remeasured ground vegetation characteristics at the nest sites to compensate for vegetation growth during the nesting season.

*Trapping and egg collection in New Mexico*

I trapped turtles from at least nine separate locations at the Bosque del Apache Refuge from 26 May until 1 July in 1999 and from 26 May to 8 June in 2000. During 1999 I used nylon-mesh hoop traps with a single funnel in one end (130 cm long; 75 cm diameter; 6.5 X 4.0 cm mesh). During 2000, I used rectangular-shaped funnel traps (30 cm high, 50 cm wide, and 80 cm long, 2.5 cm X 2.5 cm mesh). Traps were baited with a combination of sardines, canned cat food, dry cat food, chicken livers, and/or chicken gizzards. All bait was suspended in traps inside containers with small holes in their sides. Traps were left in place for 2 - 4 days and checked at least twice daily. I marked captured individuals uniquely by filing notches in their marginal scutes. Males were identified by a flatter carapace, longer claws on the front legs than on the hind legs, and the cloaca positioned posterior to the edge of the carapace. Females were larger with more domed carapaces, exhibited relatively blunt foreclaws, and the cloaca was positioned anterior to or aligned with the edge of the carapace.

Some of the smaller individuals were ambiguous; those with a smaller plastron length than the smallest identified male (85 mm) were identified as juveniles, those larger were classified as females (MacCulloch and Secoy 1983). I also intercepted and marked individuals that were encountered traversing the roads separating the refuge ponds. Eleven gravid females captured either by hand or in hoop nets during the 2000 field season were also used for collecting eggs for experimental nests. These females were injected with about 0.2 ml oxytocin subcutaneously in the anterior femoral muscle and held temporarily in plastic buckets for oviposition. All eggs were retained in moist sand in a cooler until buried in the artificial nests on 6 and 8 June.

*Construction of experimental nests in New Mexico*

I constructed experimental nests at the major nesting area at the Bosque del Apache Refuge during June 2000 to evaluate the effects of two important microhabitat variables, distance to water and canopy vegetation cover, on nest thermal and hydric conditions. This experiment consisted of constructing three nests in each block of a 2 X 2 factorial design, at 1.5 m or 15 m from the water's edge (within and outside of the range of natural nests during 1999), and under 0 or 100 percent vegetation cover from the south and west (the two most commonly sampled amounts during 1999). Cylindrical antipredator cages (Wilson 1998) of wire mesh (~ 15 cm tall, 13 cm diameter, 1 $cm^2$ grid size) were buried 13 cm into the ground, filled with surrounding soil, and the nest cavities (11 cm deep) were dug from the soil in the cages. After I placed eggs (6 per nest, selected at random from each clutch) in the cavities, I covered the nests and wired shut the tops of the cages (2 cm above the ground

surface). Four natural *C. picta* nests laid within the vicinity were not placed in antipredator cages as a control for comparing thermal profiles with experimental, caged nests.

*Temperature recording*

In New Mexico, I programmed 15 HOBO XT® temperature loggers to take temperatures ca. hourly and implanted them next to 14 painted turtle nests in 1999, and I similarly used 15 loggers to monitor the 12 experimental nests and three natural nests in 2000. The insulated wire probes extended from the loggers into the sides of the nests at a depth of about 5 cm. To examine differences in nest temperature at the top and bottom of nests I implanted two loggers at one of the nests in 1999. Loggers were implanted one to ten days after the nests were laid and recorded temperatures until 19 August during 1999 and until 8 August during 2000. Mean nest temperatures, mean maximum temperatures, and mean minimum temperatures were calculated for each nest for June, July, and August for 1999. Since many nests during 2000 experienced predation (despite the antipredator cages), nest temperatures taken during the time all nests were intact (June 8 - June 15) were used for statistical analyses.

Since 150 - 200 nests were laid during each season at the Thomson Causeway in Illinois, a subsample of the nests was selected for temperature recording that had survived until the end of June. These nests selected for sampling came from identified females, and were representative of the entire range of south and west vegetation cover over nests at the field site. Temperatures were recorded from 11 nests each in 1995 and 1999, 30 nests each in 1996 and 1997, 14 nests in 1998, and 18 nests in 2000. HOBO® XT temperature loggers, were enclosed in waterproof plastic containers and buried 1 m away from each monitored

nest, with a probe extending underground from the temperature logger into the side of the middle of the nest (Weisrock and Janzen 1999). Nest temperatures were measured ca. hourly from 18 July to 28 August in 1995, from 5 June to 30 August in 1996, from 5 June to 12 September in 1997, from 29 May to 26 September 1998, from 22 June to 22 September 1999, and from 28 June to 28 September 2000.

*Statistical analyses*

Vegetation measurements for the 30 nest sites and 40 random sites in New Mexico during 1999 were compared using Kolmogorov-Smirnov two-sample tests for differences in distributions (Sokal and Rohlf 1995). The remaining analyses were conducted using JMP 3.2.1 (SAS Institute, Inc. 1994). Mean values for randomly sampled sites versus nest sites were compared using Kruskall-Wallis non-parametric tests.

Since percentage of surface vegetation and average vegetation height were highly correlated around natural nests in New Mexico during 1999 ($N = 31$, $r^2 = 0.35$; $P < 0.001$), I combined the two measurements using a principal components analysis (Everitt and Dunn 1991) as an index of surface vegetation over a nest. The first of the two principal components described 79.6% of the total variation. The transformed variables were used as an overall quantification of the surface vegetation around nests, which was useful for reducing the number of variables used in the following regression analyses.

For each population, characteristics of natural nests were regressed on their mean July temperatures. Mean July temperatures were chosen for comparative purposes because offspring sex ratios are related to July temperatures in natural *C. picta* populations (Janzen, 1994b; Schwarzkopf and Brooks, 1985; Weisrock and Janzen, 1999). Effects of nest

characteristics on mean July nest temperatures and relative soil moisture were evaluated using multiple regression for the natural nests constructed in New Mexico during 1999. Effects of nest characteristics on nest predation were evaluated using logistic regression. Nest characteristics included: nest depth, distance from nest to water, relative soil moisture, slope, average canopy vegetation cover from the south and west, and surface vegetation for effects on July nest temperatures and predation. The same nest characteristics (excluding soil moisture) were used to evaluate relative soil moisture. In Illinois, effects of nest characteristics on mean July temperatures were also evaluated using multiple regression. The nest characteristics included: average percent canopy vegetation cover from the south and west, distances from nests to water, nest depth (for year 2000 only, since nest depths were not measured until this year), and plastron length of female (as a substitute for nest depth during all years, since these two measures were positively correlated). Non-significant predictor variables in regression analyses were excluded using stepwise elimination (Sokal and Rohlf, 1995).

Since nest survivorship for the artificial nest experiment during 2000 was too low for useful analyses, multiple regression analyses were restricted to: 1) effects of distances from nest to water and average canopy vegetation cover from the south and west on soil moisture around nests, 2) effects of distance from nest to water and average canopy vegetation from the south and west on mean nest temperatures, and 3) effects of soil moisture and average canopy vegetation cover from the south and west on mean nest temperatures. Distance from nest to water (1.5 or 15 m) and canopy vegetation cover from the south and west (0 or 100 percent) were analyzed as categorical data for the experimental nests. One datalogger could not be recovered so results for 11 experimental nests are presented.

Comparisons in nesting ecology between New Mexico and Illinois field sites included nest depth, distances from nests to water, and canopy vegetation cover over nests. To evaluate differences in nest depth, I conducted an ANCOVA using population as an effect, plastron length of females as a covariate, and nest depth as a response. Distances from nests to water and canopy vegetation cover over nests were compared using Kruskal–Wallis tests.

## Results

### *Nesting dates*

Although I searched for nests and females from 14 May through 9 July 1999, fresh nests and nesting female *C. picta* were found only between 25 May and 2 July (Julian dates 144 - 182) during the 1999 nesting season at the Bosque del Apache Refuge. Natural nests in New Mexico during 2000 were observed starting on 27 May (Julian date 147). Eight out of the 10 females captured during late May 2000 (Julian dates 147 - 151) were gravid with fully-shelled eggs, suggesting that most females had not laid their first clutch but were about to lay during late May and early June.

The nesting dates for *C. picta* in New Mexico during 1999 fell well within the range of nesting dates from Illinois turtles [Illinois: N = 6 years, start date ($\bar{x}$ ± SD): 150 ± 9 d, end date: 180 ± 3 d]. Although the end of the nesting season was not observed in New Mexico during 2000, the start date still fell within the range of dates of first nesting from Illinois turtles.

### *Nest locations and characteristics*

Painted turtle nests in New Mexico were located around the periphery of two ponds and one adjacent field that had been temporarily flooded (Figure 1). One additional nest site was also discovered by observing a nesting female next to a canal at a separate location on the refuge.

Summary statistics for seven nest characteristics measured for the 31 *C. picta* nests laid during 1999 in New Mexico and the 4 natural nests found during 2000 are displayed in Table 1, and summary statistics for Illinois nest characteristics are displayed in Table 2. Contrary to expectations, the majority of nests in New Mexico (23 out of 31) were laid under no canopy cover, and under less canopy vegetation cover from the south and west than nests from Illinois turtles during 1999 (N = 31 in New Mexico, N = 212 in Illinois, Kruskal–Wallis: $\chi 2 = 60.81$, df = 1, $P < 0.0001$). In fact, similar to Illinois turtles (Janzen and Morjan 2001), female turtles laid nests in sites with significantly lower average percentage of canopy vegetation from south and west directions than available sites (N = 30 nests in the vicinity, N = 40 sampled sites; Kruskal-Wallis test, $\chi 2 = 11.29$, df = 1, p = 0.0008, Figure 2). Results using overall average percentage canopy cover were similar (Kruskal-Wallis test, $\chi 2 = 14.09$, df = 1, p = 0.0002).

Nests and random sites in New Mexico did not differ in their means with respect to percentage of ground vegetation surrounding the sites (Kruskal-Wallis test, $\chi 2 = 0.50$, df = 1, p = 0.48) and mean vegetation height (Kruskal-Wallis test, $\chi 2 = 0.77$, df = 1, p = 0.38) in New Mexico, although distributions of all three vegetation characteristics (average % canopy cover, % ground vegetation surrounding nests, and average vegetation height) differed significantly between nest sites and random sites (Figure 2).

Notably, painted turtles in New Mexico laid nests much closer to the water than did Illinois turtles (nests laid during 1999: New Mexico, N = 30, $\bar{x} \pm$ SD: 2.4 ± 2.2; Illinois, N = 206, $\bar{x} \pm$ SD: 31.5 ± 23.7; Kruskal–Wallis $\chi^2 = 53.38$, df = 1, $P < 0.0001$; Tables 1 and 2). During 1999, distance from nests to water was negatively correlated with soil moisture levels at nest depth (11 cm) in New Mexico ($r^2 = 0.25$, $F_{(1,28)} = 9.06$, $P = 0.0056$). No females during their nesting forays (or tracks left from nesting forays) were observed to wander more than a few meters from edges of all the ponds in this population. Nests surrounding the ponds were typically placed within a visible ring of darker soil made from increased soil moisture around the ponds, which had a width of ca. 2 - 3 m, the average distance from nests to the water (pers. obs.). This observation was corroborated by the fact that relative soil moisture at nest depth decreased dramatically between nest sites and at sites only one m beyond the nests, perpendicular to the water line (paired t-test: $n = 30$, $t = 4.68$, $P < 0.0001$), suggesting that females preferentially placed nests in this narrow available range of moist conditions. Relative soil moisture at nests in New Mexico during 1999 also increased from mid-nest depth (5.5 cm) to nest depth (11 cm) (paired t-test: $N = 30$, $t = 4.46$, $P < 0.0001$).

Nests in New Mexico were relatively deeper than were *C. picta* nests in Illinois (Table 3). Differences in female sizes were not a cause for the differences in nest depths between populations. Nest depth increased with plastron size for both populations, suggesting that larger females lay deeper nests (Table 3). Nevertheless, significant differences in nest depths still existed across populations after accounting for effects of female size (Table 3), indicating that nests were disproportionately deeper for female size in New Mexico. Since I did not have information on most of the females that laid the nests in

New Mexico, and for some of the females in Illinois, I also conducted a similar ANCOVA using clutch size as a correlate for female size rather than plastron length to increase total sample sizes by 23 nests. Effects of population, clutch size, and their interaction on nest depth were the same as using plastron length in the smaller analysis (data not shown).

*Nest microhabitat and predation*

Nests placed on steeper slopes were less likely to experience predation in New Mexico during 1999 (logistic regression: N = 31; likelihood-ratio $\chi^2 = 7.46$, P = 0.0063). No other microhabitat characteristics, including distance from nest to water, average percent canopy vegetation, soil moisture, or surface vegetation, were associated with nest predation (likelihood-ratio $\chi^2_{(1,21)}$: 1.49, 1.84, 2.16, 0.02; P = 0.22, 0.18, 0.14, and 0.90, respectively).

*Thermal conditions of natural nests*

Summary statistics for *C. picta* nest temperatures taken from 12 nests in New Mexico during June - August 1999 are displayed in Table 4 (dataloggers failed for the two remaining nests). During 2000, three of the four natural nests were depredated by mid-June so summary data are not shown for these nests. New Mexico nests contained on average ten eggs, with most laid in a single layer at the bottom of the nest, so I expected little within-clutch variation in nest temperatures. Mean July temperatures within a New Mexico nest in which temperatures were taken at the top and bottom of the nest cavity during 1999 differed by 0.5°C, far within one standard deviation of mean temperatures experienced among clutches (Table 4). Therefore, for the following analyses, I focused on among-clutch temperature variation rather than within-clutch variation in nest temperatures.

No microhabitat variables measured from the 12 natural nests in New Mexico during 1999, including nest depth, distance to water, soil moisture, surface vegetation, or various interactions were correlated with mean maximum, mean minimum, or mean July nest temperatures (multiple regression: $P > 0.50$ for all; Table 5). This lack of relationship may be due to a lack of variation in several of microhabitat characteristics that may be important for nesting in this population. For example, 10 of the 12 nests were laid under 0% canopy vegetation, and 11 of the 12 were located less than 3 m from the water. The experimental nests constructed during 2000 were likely more effective than an observational approach to evaluate the effects of the two major nesting patterns that I observed (short distances of nests to water and low amounts of canopy vegetation over nests) on nest thermal and hydric conditions.

In Illinois, three variables were correlated with mean July nest temperatures: year, percentage of canopy cover over nests from the south and west, and female plastron length ($r^2 = 0.73$, Table 6). Since nest depth increased with female plastron length during 2000 (90 nests, $r^2 = 0.17$, $F_{1,88} = 18.27$, $P < 0.0001$), I used plastron length as a substitute for nest depth to evaluate its effects over the six years of the study. Nest depth, had it been measured prior to 2000, would have likely been a significant influence on nest temperatures as deeper nests were cooler during 2000 (16 nests: percent vegetation cover from south and west: $F_{1,13} = 18.19$, $P = 0.0009$, nest depth: $F_{1,13} = 6.03$, $P = 0.029$), and nests from larger females tended to be cooler during all years. Distance to water (Table 6) and interaction effects among the measured variables (not shown) were not correlated with nest temperatures.

*Thermal and hydric conditions for experimental nests*

Natural nests in New Mexico, which were unshaded and close to the water, experienced similar mean thermal conditions as experimental nests in the same environmental zone (Figure 3). Similar to natural nests from 1999, distance from nest to water negatively affected soil moisture surrounding nests (Table 7). Additionally, canopy vegetation cover positively affected soil moisture (Table 7). Nest temperatures were negatively correlated with canopy vegetation cover, soil moisture, and their interaction ($r^2$ = 0.97, Table 8). The interaction effect between soil moisture and canopy cover indicated that low soil moisture was associated with a dramatic increase in nest temperatures for those placed in the open, but not for nests placed in the shade (Figure 3). For example, for nests under no vegetation cover from which July temperatures were available, average July temperatures for those placed far from the water were 29 and 30°C, which were 3-4°C hotter than those placed near the water and likely to be lethal to developing embryos. Moist soil conditions likely reduced mean nest temperatures through evaporative cooling rather than insulating nests, as higher soil moisture at nests was associated with lower mean daily maximum temperatures during the day (regression: $r^2 = 0.99$, P = 0.01), but only weakly associated with higher mean daily minimum temperatures at night (regression: $r^2 = 0.40$, P = 0.09). Distance from nest to water or its interaction with canopy vegetation cover did not significantly affect mean nest temperatures and were ruled out of the analysis using stepwise regression (P = 0.38 and P = 0.68, respectively).

Mean July temperatures for nests at the Bosque del Apache Refuge fell within the range of mean July temperatures measured for *C. picta* nests in Illinois (Figure 4), despite

the Bosque Refuge experiencing warmer July temperatures than the 50-year average during both 1999 and 2000 (National Oceanic and Atmospheric Administration, 1999, 2000).

*Sex ratios of captured adults in New Mexico*

Nearly equal numbers of male and female painted turtles were captured at the Bosque del Apache Refuge by hoop traps and by hand during 1999 and 2000 (71 males, 70 females, 5 juveniles). No differences among years in sex ratio existed: 45 males and 45 females were captured during 1999, and 26 males and 25 females were captured during 2000. Although several recaptures occurred within years, only 4 turtles from 1999 were recaptured in 2000.

## Discussion

Theoretical models identify heritable variation in maternal nesting related to thermal qualities and thermal response of offspring sex as two important mechanisms for sex ratio evolution in TSD (Karlin and Lessard 1986, Bulmer and Bull 1982, Bull 1983). Pivotal temperatures exhibit little variation among populations with TSD, and do not tend to vary in patterns concordant with large-scale climatic conditions (Ewert et al. 1994). Although variation in and selection on nesting behavior by females has been thought to be important for the evolution and maintenance of sex ratios in reptiles with TSD (Bull et al., 1982a; 1982b; Ewert et al., 1994), no direct comparisons had been made prior to this study. Painted turtles in New Mexico and Illinois nest at similar times; thus, nests in New Mexico experience climatic conditions expected to produce 100% females for their northern conspecifics (Janzen 1994a). Yet nests in New Mexico did not experience hotter mean

temperatures, even when considering that the climatic conditions at the field site were hotter than average during the study (National Oceanic and Atmospheric Administration et al. 1999, 2000).

Since vegetation surrounding nests reduces nest temperatures (Vogt and Bull 1984, Roosenburg 1996, Wilson 1998, Weisrock and Janzen, 1999), and casual observations suggest that females may nest under greater amounts of cover in the south than in the north (Ewert, 1976; Janzen and Morjan, 2001; Vogt and Flores-Villela, 1992; Wilson, 1998), *C. picta* females were expected to lay nests under relatively greater vegetation cover in New Mexico to compensate for hotter climatic conditions. On the contrary, females laid nests not only under less canopy cover than their more northern conspecifics, but under less canopy vegetation than available to them at the site. This pattern occurred despite greater amounts of canopy vegetation being associated with reduced nest temperatures.

Instead, nesting patterns differed in that nests in New Mexico were deeper and placed closer to the water than nests in Illinois. Both factors are likely to increase water availability in natural nests. Cagle et al. (1993) found that water potential affected hatching success, hatchling size, and hatchling mass more than did temperatures in natural *C. picta* nests, similar to laboratory results (Packard et al. 1989). Additionally, Packard and Packard (2000; 2001) noted that mean nest temperatures explained relatively minor amounts of variation in *C. picta* hatchling mass and fat reserves compared to hydric conditions of the nests. They noted, however, that mean temperatures of nests may not be representative of the thermal profiles all eggs experience within the nest. Despite the relative importance of nest hydric conditions on hatchling phenotypes, previous field studies on nest-site selection in painted turtles suggest that nest-site selection may instead be based on thermal qualities:

females tend to nest in areas likely to be warmer than random or available sites in Illinois and Ontario (Schwarzkopf and Brooks 1987, Janzen and Morjan 2001, but see Morjan and Valenzuela 2001), and individual females in Illinois tend to repeatedly select sites with vegetation characteristics associated with thermal qualities of nests rather than with hatching success, predation, or hydric conditions (as evaluated through relative hatchling mass) (Janzen and Morjan 2001). The majority of *C. picta bellii* nests in Illinois (Janzen and Morjan 2001) and northwestern Iowa (Ratterman and Ackerman 1989) are constructed tens of meters from standing water, whereas nests in New Mexico were constructed only a few meters from the water. This major difference in nesting ecology underscores the importance of hydric conditions in nest-site selection in arid conditions in New Mexico, as distance from nests to water was not correlated with predation or directly with nest temperatures, but rather with nest hydric conditions. Since water levels at the Bosque del Apache Refuge fluctuate substantially, this pattern of nesting likely reflects a steep trade-off between the risks of flooding (known to cause mortality for at least 3 nests) and desiccation for eggs and hatchlings traveling from nests to water.

The observed patterns of nest-site selection simultaneously places nests in intermediate thermal conditions relative to nests placed in less frequently used areas (Figure 3). The artificial nest experiment demonstrated that exposed nests placed close to the water were more likely to experience lower temperatures than exposed nests placed at greater distances from the water. Eleven out of the 12 natural nests from which temperatures were taken during 1999 were exposed nests close to the water, which experienced the same thermal range of both natural and experimental nests placed in the same two microhabitat categories during 2000 (Table 4; Figure 3). These results indicate that by maximizing water

availability at nests by nesting close to standing water, females are also nesting in areas likely to reduce nest temperatures. Nesting patterns of *C. picta* in New Mexico demonstrate that although nests experience similar thermal conditions as those from their conspecifics in cooler climates, in this population, hydric conditions of nests may primarily influence nest-site selection.

Regardless of the proximate mechanisms driving nest-site selection among New Mexico and Illinois *C. picta*, if nesting patterns in New Mexico did not provide similar thermal conditions, there likely would be an extremely female-biased primary sex ratio in New Mexico likely to be reflected in the adult population sex ratio (if the population would exist at all). Although evaluating sex ratios for adult turtles presents difficulties (e.g., Ream and Ream, 1966; Thomas et al. 1999), it is clear that an adult male population in New Mexico is thriving and perhaps equal in number to females. Longer-term trapping efforts at the Bosque del Apache Refuge, including pre- and post- nesting seasons, also suggest a nearly 1:1 sex ratio in that population (J. N. Stuart, pers. comm.). Since females in New Mexico nest at similar times as their northern conspecifics, pivotal temperatures in New Mexico are only slightly higher than Illinois pivotal temperatures, and nests experience similar temperatures between New Mexico and Illinois populations during egg incubation, geographic variation in nesting patterns is likely an important cause for the adequate numbers of males being produced in New Mexico.

Nevertheless, such geographic variation in nesting patterns may not be adaptive in terms of sex ratio evolution. When examining patterns of geographic variation in nest placement for reptiles with TSD, other factors influencing nest-site selection should be considered since fitness consequences for placing nests in environments likely to produce

the sex in greater demand seem relatively weak compared to selection for nest-sites maximizing offspring survivorship. Schwarzkopf and Brooks (1987) concluded that female *C. picta* in Ontario consistently chose relatively warm sites in a unimodal thermal distribution to ensure completion of egg development rather than to influence offspring sex ratios. Similarly, Ewert et al. (1994) suggested that geographic variation in nest-site selection by females may reflect local adaptations for maximizing developmental rate and hatching success of eggs. Variation in pivotal temperatures may instead reflect sex ratio selection across population differences in nest microclimates, which may not differ substantially across varying larger-scale climatic conditions (Ewert et al. 1994). This study supports this hypothesis by Ewert et al. (1994) because of low variation in *C. picta* pivotal temperatures among populations (Ewert et al., 1994), but nesting patterns by female *C. picta* in New Mexico are directly related to nest hydric conditions but only indirectly related to thermal conditions. Consequently, adaptive interpretations for maternal behavior influencing nest temperatures in reptiles with TSD require evaluation within the context of other selective pressures.

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**TABLE 1.** Attributes of *Chrysemys picta* nests during 1999 (N= 17 - 32) and 2000 (N = 4) at Bosque del Apache National Wildlife Refuge, Soccorro County, New Mexico. Clutch size and nest depth are reported for intact nests during 1999. Distance from nest to water was not measured for one nest during 1999 because the water receded substantially overnight between nesting and measurement.

| | 1999 | | | 2000 (N=4) | |
|---|---|---|---|---|---|
| Nest attribute | ($\bar{x}$ ± SD) | Range | N | ($\bar{x}$ ± SD) | Range |
| Clutch size | 10.1 ± 2.2 | 6-14 | 17 | 9.0 ± 1.6 | 7 - 11 |
| Nest depth (cm) | 11.4 ± 0.9 | 10-13 | 17 | 10.8 ± 1.0 | 10 - 12 |
| Distance to water (m) | 2.4 ± 2.2 | 0.7 - 11.4 | 30 | 1.67 ± 0.64 | 0.99 - 2.40 |
| Slope (degrees) | 10.4 ± 5.6 | 0 - 23 | 31 | 9.5 ± 5.0 | 4 - 16 |
| % SW canopy vegetation | 7.2 ± 21.0 | 0.0 - 97.0 | 31 | 19.1 ± 26.3 | 0.3 - 57.8 |
| Percent ground vegetation | 40.3 ± 31.6 | 0-90 | 31 | 72.5 ± 48.6 | 0 - 100 |
| Average plant height (cm) | 11.7 ± 8.3 | 0-31 | 31 | 12.5 ± 10.1 | 0 - 21.6 |

**TABLE 2.** Attributes of *Chrysemys picta* nests from 1995 - 2000 at the Thomson Causeway Recreation Area in Carroll Co., Illinois. Distances from nests to water were measured from 1997 - 2000, and nest depth was measured during 2000.

| Nest attribute | ($\bar{x} \pm$ SD) | Range | N |
|---|---|---|---|
| Nest depth (cm) | 8.7 ± 0.8 | 6.0 - 10.9 | 129 |
| Distance to water (m) | 30.1 ± 23.1 | 0 - 87.5 | 739 |
| % SW canopy vegetation | 44.6 ± 22.2 | 0 - 99.0 | 1023 |

**TABLE 3.** Effects of population and plastron length on depths of natural *Chrysemys picta* nests in New Mexico during 1999 (n = 10) and Illinois during 2000 (n = 87). All repeated nesting events from the same females were excluded.

| Effect | F | df | P |
|---|---|---|---|
| Population | 109.30 | 1 | < 0.0001 |
| Plastron length | 16.57 | 1 | < 0.0001 |
| Population X plastron length | 1.24 | 1 | 0.27 |

**TABLE 4.** Summary statistics of nest temperatures ($\bar{x}$ ± SD, range) for natural painted turtle nests at Bosque del Apache National Wildlife Refuge during the 1999 nesting season.

| Month | Mean (°C) | Mean minimum (°C) | Mean maximum (°C) |
|---|---|---|---|
| June | 25.0 ± 0.51 | 19.9 ± 0.75 | 31.7 ± 2.07 |
| (n = 5) | 24.37 - 25.70 | 18.74 - 20.61 | 28.92 - 34.59 |
| July | 25.97 ± 1.35 | 22.40 ± 0.96 | 30.77 ± 2.50 |
| (n = 12) | 23.95 - 28.23 | 21.21 - 24.21 | 27.30 - 34.71 |
| August | 24.62 ± 1.30 | 21.61 ± 1.35 | 29.07 ± 5.08 |
| (n = 10) | 21.94 - 26.2 | 18.57 - 23.08 | 23.76 - 41.63 |

**TABLE 5.** *F*-ratios (df = 1,11) and levels of significance for nest microhabitat effects on mean maximum, mean minimum, and mean July nest temperatures for 12 natural *Chrysemys picta* nests at the Bosque del Apache National Wildlife Refuge, New Mexico during 1999, evaluated through stepwise elimination. Surface vegetation was a principal-component score combining percentage of surface vegetation surrounding nests and vegetation height. SW canopy vegetation was the average percentage of canopy vegetation over the nest in the south and west directions.

| Source of variation | Mean maximum | Mean minimum | Mean |
|---|---|---|---|
| Nest depth | 0.09 | 0.43 | 0.02 |
| | (0.77) | (0.52) | (0.89) |
| Distance to water | 0.88 | 2.49 | 1.17 |
| | (0.37) | (0.15) | (0.31) |
| Soil moisture | 1.79 | 0.07 | 0.18 |
| | (0.21) | (0.80) | (0.68) |
| Surface | 0.00 | 0.00 | 0.12 |
| vegetation | (1.00) | (1.00) | (0.74) |
| SW canopy | 0.93 | 1.73 | 1.01 |
| vegetation | (0.36) | (0.22) | (0.34) |

**TABLE 6.** Effects of distance to water and plastron length of females on mean July temperatures in a multiple regression using data from 108 natural nests of *Chrysemys picta* in Thomson, Illinois, from 1995 - 2000 ($r^2 = 0.73$). Plastron length was used as a correlate for nest depth, as larger females laid deeper nests during 2000 (see Results). Effects of year and percent vegetation cover from the south and west on mean July nest temperatures in this population for 1995 - 1999 were previously evaluated by Morjan (2002).

| Effect | Estimate | SE | F | DF | P |
|---|---|---|---|---|---|
| Year | | | 38.26 | 5 | < 0.0001 |
| % SW canopy vegetation | -0.034 | 0.0040 | 72.84 | 1 | < 0.0001 |
| Plastron length | -0.021 | 0.011 | 3.85 | 1 | 0.053 |
| Distance to water | -0.0012 | 0.0042 | 0.074 | 1 | 0.79 |

**TABLE 7.** Effects of canopy vegetation cover from the south and west (0 or 100 percent) and distance from nests to water (2 or 15 m) on soil moisture surrounding 12 experimental nests of *Chrysemys picta* in New Mexico ($R^2 = 0.77$).

| Effect | Estimate | SE | F | DF | P |
|---|---|---|---|---|---|
| Shade category | 2.89 | 1.19 | 5.88 | 1 | 0.0383 |
| Distance category | -5.96 | 1.19 | 25.00 | 1 | 0.0007 |
| Shade X Distance | -0.60 | 1.25 | 0.23 | 1 | 0.64 |

**TABLE 8.** Effects of canopy vegetation cover from the south and west (0 or 100 percent) and soil moisture on mean *Chrysemys picta* nest temperatures taken from 11 nests during 5 - 15 June, 2000, in New Mexico ($R^2 = 0.97$).

| Effect | Estimate | SE | F | DF | P |
|---|---|---|---|---|---|
| Shade category | -3.63 | 0.35 | 105.96 | 1 | < 0.0001 |
| Soil moisture | -0.070 | 0.027 | 6.95 | 1 | 0.034 |
| Shade category X Soil moisture | 0.081 | 0.027 | 9.33 | 1 | 0.019 |

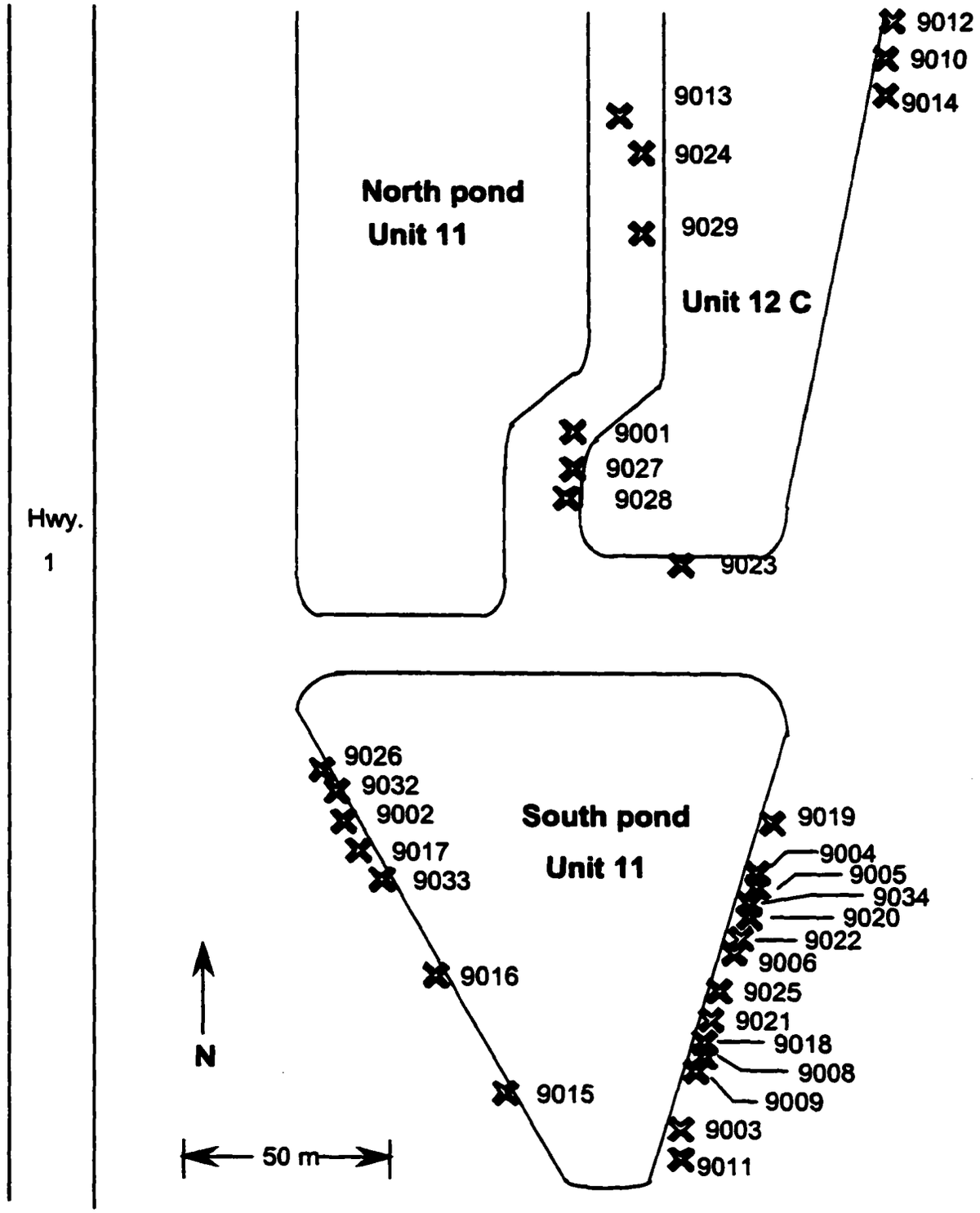
**Figure 1.** Locations of *Chrysemys picta* nests at the north and south ponds of Unit 11 and 12C at Bosque del Apache National Wildlife Refuge, San Antonio, New Mexico, during 1999. Nest 9001 was a successfully hatched nest from 1998 containing 3 dead hatchlings, not used in analyses for 1999 data. One additional *C. picta* nest at a farther location from Unit 11 is not shown.

**Figure 2.** Distributions of average percentage canopy vegetation (from all directions), average percentage of ground vegetation, and average vegetation height for 30 nest sites versus 40 randomly sampled sites at Bosque del Apache National Wildlife Refuge, New Mexico. Results of Kolmogorov-Smirnov two-sample tests for differences in these distributions are displayed on the left, and means ± SD with median values are displayed in the boxes for each distribution.

**Figure 3.** Mean nest temperatures taken during June from natural and experimental nests of *Chrysemys picta* eggs at the Bosque del Apache National Wildlife Refuge, New Mexico. Soil moisture exhibited a bimodal distribution with a median value of 15%. Filled circles depict nests with soil water content higher than 15% of its mass, open circles depict nest sites with soil water content less than 15%.

**Figure 4.** Mean July temperatures for *Chrysemys picta* nests during 1999 - 2000 at the Bosque del Apache National Wildlife Refuge in New Mexico (black circles), and during 1995 - 2000 at the Thomson Causeway Recreation Area in Illinois (white circles). The sole surviving experimental nest located in similar microhabitat to natural nests was included for

New Mexico in 2000 because nest survivorship was low.


Hwy.
1
North pond
Unit 11
Unit 12 C
South pond
Unit 11
9012
9010
9014
9013
9024
9029
9001
9027
9028
9023
9026
9032
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9015
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9004
9005
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9006
9025
9021
9018
9008
9009
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9011
N
50 m

**Figure 1.**

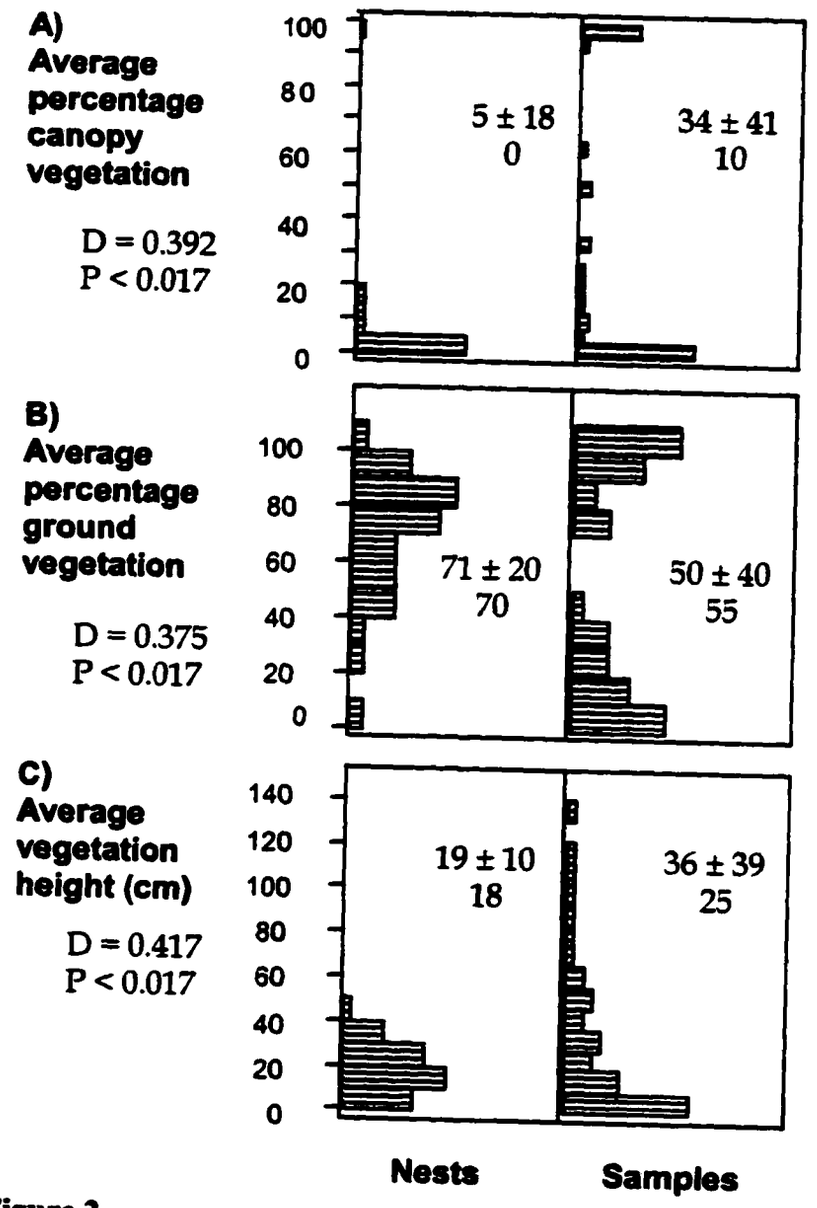
A)
Average
percentage
canopy
vegetation
D = 0.392
P < 0.017
100
80
60
40
20
0
5 ± 18
0
34 ± 41
10
B)
Average
percentage
ground
vegetation
D = 0.375
P < 0.017
100
80
60
40
20
0
71 ± 20
70
50 ± 40
55
C)
Average
vegetation
height (cm)
D = 0.417
P < 0.017
140
120
100
80
60
40
20
0
19 ± 10
18
36 ± 39
25
Nests
Samples

**Figure 2.**

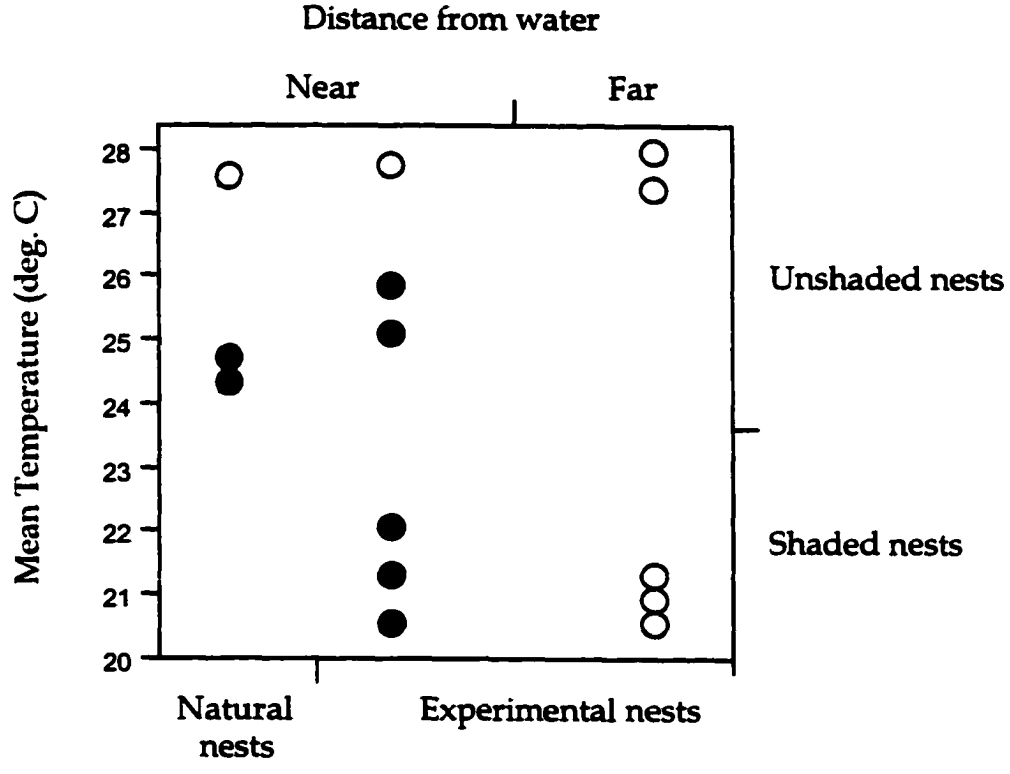
Distance from water
Near
Far
Mean Temperature (deg. C)
28
27
26
25
24
23
22
21
20
Unshaded nests
Shaded nests
Natural nests
Experimental nests

**Figure 3.**

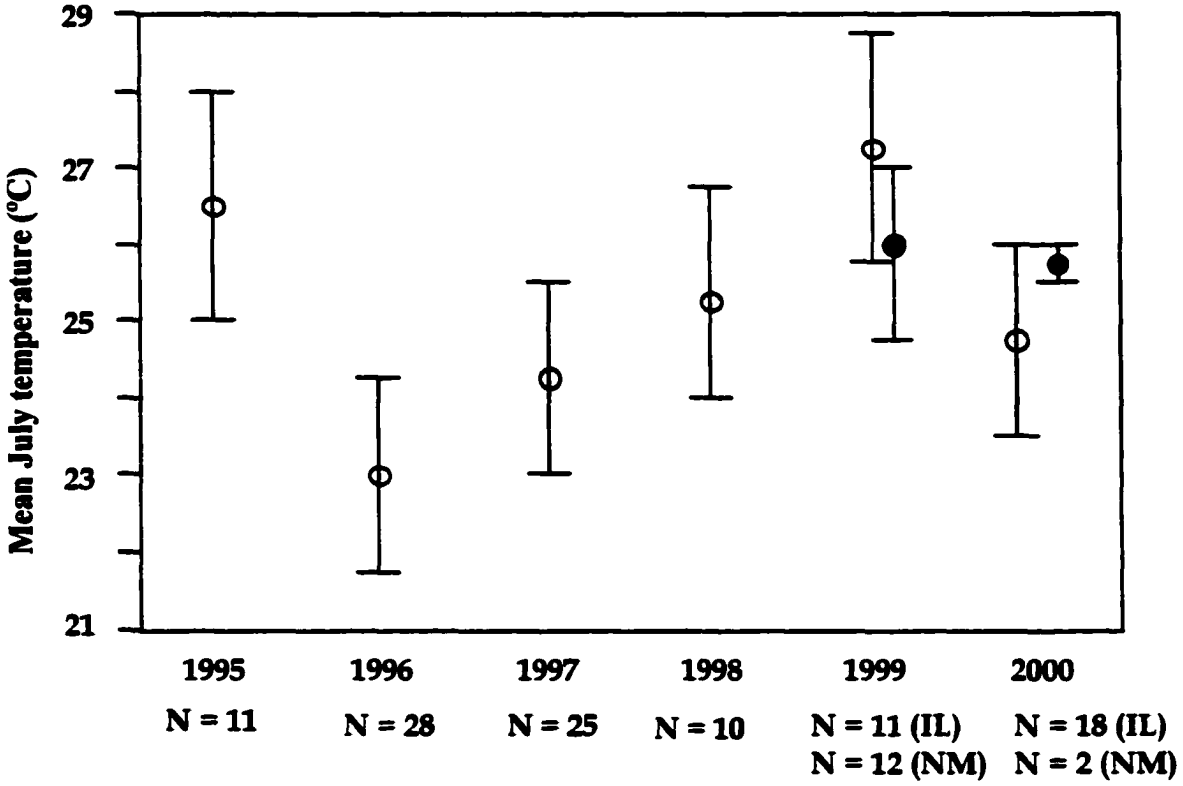
Mean July temperature (°C)
29
27
25
23
21
1995
N = 11
1996
N = 28
1997
N = 25
1998
N = 10
1999
N = 11 (IL)
N = 12 (NM)
2000
N = 18 (IL)
N = 2 (NM)

**Figure 4.**

# CHAPTER 5.

## How rapidly can maternal behavior affecting primary sex ratio evolve in reptiles with environmental sex determination?

A paper submitted to American Naturalist

Carrie L. Morjan

Department of Zoology and Genetics

Iowa State University

Ames, IA 50011

## ABSTRACT

Theoretical models identify an important role of maternal behavior in the maintenance and evolution of sex ratios in organisms with environmental sex determination (ESD). Maternal choice of oviposition site is generally thought to respond more rapidly to sex ratio selection than environmental sensitivity of offspring sex in reptiles with temperature-dependent sex determination (TSD). However, knowledge of the evolutionary potential for either of these traits in a field setting is limited. I developed a simulation model using local climate data and observed levels of phenotypic variation for nest-site choice and thermal sensitivity of offspring sex ratios in painted turtles (*Chrysemys picta*) with TSD. Both traits, and hence sex ratios, evolved slowly to simulated climate change scenarios. In contrast to expectations from previous models, nest-site choice evolved more slowly than thermal sensitivity of offspring sex due to large climatic effects on nest temperatures and indirect selection on maternally-expressed traits. A variant of the model, assuming inheritance of nest-site choice through natal imprinting, demonstrated that natal imprinting inhibited adaptive responses in

female nest-site choice to climate change. These results predict females have relatively low potential to adaptively adjust sex ratios through nest-site choice in this system.

**KEYWORDS:** temperature-dependent sex determination, sex ratio evolution, maternal effects, climate change, turtles

## Introduction

Maternal effects have important, although sometimes nonintuitive, evolutionary consequences (Kirkpatrick and Lande 1989; Wade, 1998). Among these affects is maternal behavior affecting offspring phenotypes, which likely plays an important role in adaptation and evolutionary dynamics of populations. In particular, oviposition-site choice by females coupled with progeny response to their provided environment is an example of how maternal and offspring traits may interact to produce a single phenotype. The coevolution of these two traits may lead to relatively rapid local adaptation through runaway selection (Wade, 1998). For example, in reptiles with temperature-dependent sex determination (TSD), selection on maternal and offspring traits affecting primary sex ratio is likely important for maintaining sex ratios in changing thermal environments (Bulmer and Bull 1982, Bull 1983). However, which of the two traits is more likely to respond to selection has been long debated (Bull et al. 1982a, 1982b; Janzen 1992, Rhen and Lang, 1998, Janzen and Morjan 2001), but environmental variation mediating these traits has played a key role in these discussions (e.g. Bull et al. 1982a, Janzen 1992).

Despite the fact that temperature-dependent sex determination (TSD) was discovered in reptiles more than 35 years ago (Charnier 1966), its proximate mechanisms, adaptive

significance, and potential for sex ratio evolution remain unclear (Bull 1980; Bull et al. 1982a; Pieau et al. 1999; Shine 1999). Although TSD has persisted in ancient taxa which have undoubtedly endured substantial environmental upheaval (Janzen and Paukstis 1988, 1991a,b), changes in egg incubation temperatures as small as 1-2°C drastically skew offspring sex ratios in the laboratory (Bull 1980; Ewert and Nelson 1991) and in the field (Bull 1985; Janzen 1994a; Weisrock and Janzen 1999). The only long-term data correlating local climatic condition with annual cohort sex ratios in a reptile with TSD suggest that skewed primary sex ratios are likely to result from rapid and relatively small climatic changes ($\leq$ 2°C; Janzen, 1994a). Such skewed sex ratios, should they persist to adulthood, are thought to be maladaptive, as genes causing individuals to produce the rarer of the two sexes increase in frequency through frequency-dependent selection. (Fisher 1930).

Theoretical models identify two mechanisms important for the microevolution of sex ratios in populations exhibiting TSD: maternal nest-site choice related to nest thermal properties, and variation in offspring sex ratios in response to thermal environment (Bulmer and Bull 1982; Charnov 1982; Bull 1983; Karlin and Lessard 1986). Although substantial among-clutch variation in offspring sex ratio in response to temperature exists for several species exhibiting TSD, its significance for microevolution of sex ratio has been thought to be relatively low (Bull et al. 1982a; Janzen 1992; but see Rhen and Lang 1998). Eggs incubated at constant temperatures in the laboratory exhibit high heritabilities for sex ratio, but effective heritabilities for sex ratio in field-incubated nests are severely diminished due to environmental sources of sex ratio variation (0.03 – 0.06; Bull et al. 1982a; Janzen 1992). Maternal choice of thermal environments for nests has thus been thought to be more likely

to respond to selection than embryonic response to temperature in organisms with TSD (Bulmer and Bull 1982; Bull et al. 1982a).

Few studies have addressed differences among individual females in nest-site selection, however. Such information would be critical to evaluate whether selection may act on such differences in the maintenance and evolution of sex ratios. In one study, females from a laboratory colony of leopard geckoes (*Eublepharis macularius*) exhibiting TSD did not differ statistically in their choice of nest temperatures, although an upper limit of the heritability for nest temperature was estimated to be as high as 0.20 (Bull et al. 1988). In a second laboratory study, Bragg et al. (2000) detected significant differences in nest-site temperatures among individual female *E. macularis* and in a second species of gecko exhibiting TSD (*Hemitheconyx caudicinctus*). More recently, female painted turtles (*Chrysemys picta*) were observed to exhibit a repeatability of nest-site selection ($r$ = 0.18 - 0.21) to microhabitats affecting nest temperatures and offspring sex ratios in a natural population with TSD (Janzen and Morjan, 2001). Repeatability estimates are thought to provide an upper estimate of the heritability for the trait (Arnold 1994; Boake 1989); however, calculating actual heritabilities for nest-site choice in this population through breeding would be quite difficult in this system (Janzen and Morjan, 2001). Compared to the relatively low estimates of effective heritabilities for offspring sex ratios in reptiles with TSD, the heritability of nesting behavior affecting nest thermal qualities may not need to be much higher to respond more rapidly to selection.

Long-term data on a population of painted turtles *C. picta* provide the opportunity to model the relative evolutionary potential of these maternal and offspring traits. *Chrysemys picta* is a common freshwater turtle that inhabits a wide latitudinal range across varying

climatic regimes (Ernst et al. 1994), although annual offspring sex ratios within an Illinois population vary substantially with small changes in summer climatic conditions (Janzen 1994a). Females vary significantly in their choice of microhabitat over nest-sites in this population (Janzen and Morjan 2001), which is correlated with nest temperatures and offspring sex ratios (Janzen 1994b, Weisrock and Janzen 1999). In this study, I developed a simulation model based on population parameters of *C. picta* to evaluate the relative evolutionary potential for nest-site choice and thermal sensitivity of offspring sex, while including substantial levels of thermal variation in field-incubated nests observed both within and among nesting seasons. Two variations of the model were constructed, one assuming an additive genetic basis for both nest-site choice and thermal sensitivity of offspring sex, and the other assuming inheritance of nest-site choice through natal imprinting to nest microenvironments.

## Methods

### *Study organism and focal population*

Data were derived from a well-studied nesting population of painted turtles (*Chrysemys picta*) on an island in the Mississippi River from 1988 through 1999 (Janzen 1994a,b; Weisrock and Janzen 1999; Janzen and Morjan 2001; Valenzuela and Janzen 2001). Annual offspring sex ratios are highly correlated with mean July air temperatures experienced at the site during the egg incubation period (Janzen 1994a; F.J. Janzen unpubl. data). Within years, offspring sex ratios in this population are also correlated with percentage of canopy vegetation cover over nests from the south and west directions

(Janzen, 1994b). However, this correlation exists only during years experiencing typical climatic conditions; extreme climatic conditions over-ride the effects of vegetation cover on offspring sex ratios, producing unisexual or nearly-unisexual yearly cohorts (Janzen 1994a,b).

Within years, nest temperatures are inversely correlated with the percentage of canopy vegetation cover over the nests, such that nests under high vegetation cover tend to be cooler (Weisrock and Janzen 1999; Morjan and Janzen, unpubl. data). Although variation in mean July air temperatures affects the intercept of the linear relationship between canopy vegetation and nest temperatures, the slopes remain parallel across years (Morjan and Janzen, unpubl. data). Nesting females at this site are repeatable in nest-site choice in terms of vegetation cover over nests, which is not explained by nest-site fidelity on a geographic scale (Janzen and Morjan 2001; Valenzuela and Janzen 2001).

Details for collecting and incubating *Chrysemys picta bellii* eggs from the study site in Illinois during 1998 are described in a separate study evaluating among-population variation in sex determination (Morjan 2002). Offspring sex ratios as a function of clutch and temperature are displayed in Table 1. The shape parameter of the response curve of sex ratio to temperature (*S*) for the focal population was calculated using maximum likelihood (Program 1.3 for Mac, Girondot 1999). Data available from the literature were also used to calculate *S* for two other populations of C. picta located in Wisconsin and Tennessee (Bull et al. 1982b). The model used describes the function in which the frequency of males (*sr*) is produced at a constant temperature, t:

$$sr(t) = \frac{1}{1 + e^{\left(\frac{1}{S}(P-t)\right)}}, \quad (1)$$

where $P$ is the pivotal temperature (the constant laboratory temperature producing a 1:1 sex ratio) and $S$ describes the shape of the transition in sex ratios across temperatures (Girondot 1999). Higher absolute values of $S$ represent a wider transition range of temperatures producing both sexes (Chevalier et al. 1999); therefore higher $|S|$ suggests a wider range of temperatures where genetic variation in sex ratios can be expressed.

## *The model*

*Model development and life cycle*

The yearly life cycle for the simulation model is depicted in Figure 1. Modelling nest temperatures within and across years is discussed as follows. Mean July air temperatures and the percentage of vegetation cover from the south and west over the nest were significant sources of variation for 94 nest temperatures taken from 1995 – 1999 [multiple regression, $F_{(2,91)} = 122.77$, $r^2 = 0.73$, $P < 0.0001$]. Therefore, these two variables were used to derive a model for nest temperature of nest $j$ ($T_{yj}$) as a linear function of yearly climatic condition ($C_y$) for year $y$ and microhabitat of the nest ($N_j$):

$$T_{yj} = \alpha + \beta C_y + \gamma N_j + \varepsilon \quad (2)$$

where $\alpha$ and $\varepsilon$ are the intercept and an error effect, respectively.

Threshold temperature was defined as the temperature which, when exceeded, caused an individual to become female. This term is not to be confused with pivotal temperature, which describes the temperature producing a 1:1 sex ratio of a clutch or

population (Mrosovsky and Pieau 1991), rather than an attribute of an individual. For the purposes of this study, pivotal temperature is defined as the mean threshold temperature for the population. Within clutches (defined to consist of eight offspring), each individual's genetically inherited threshold temperature ($Z_i$), was compared to the nest temperature experienced by that clutch. If the nest temperature experienced by a clutch was higher than an individual's threshold temperature, the individual was recruited as a female into the adult population, otherwise it was recruited as a male (Figure 1).

Individual survivorship varied across two life stages: offspring survivorship to year 1, and adult survivorship. Survivorship rates for males and females were equal within all life stages. The breeding population consisted of all males of at least age 3 and all females of at least age 5, equal to estimated ages of first reproduction for painted turtles in the study population (F. J. Janzen, pers. comm.). Each female mated with a randomly selected male and produced one clutch of eggs per year. During years of female-biased sex ratios, a marriage function determined the fraction of females [$B(n_f)$] that bred during that year:

$$B(n_f) = \frac{2n_m}{n_m + n_f} \tag{3}$$

where $n_m$ is the number of males and $n_f$ is the number of females in the breeding population. The harmonic mean was chosen because it is considered to be less problematic than using simply a female-dominant marriage function in two-sex models (Caswell and Weeks 1986; Caswell 1989). Although likely having no biological basis in turtles, this function was useful for the simulations because it prevented exploding population growth and crashing simulations during years of highly female-biased sex ratios. However, no qualitative differences in relative rates of evolution of nest-site choice and threshold temperatures, or

the rate of sex ratio evolution, occurred whether or not the function was included in the simulations (C. Morjan, unpubl. data).

*Inheritance of Z and N*

The mode of inheritance of threshold temperature (Z) and nest-site choice (N) is based on an earlier model of additive genetic variation for threshold temperatures by Bull and Bulmer (1989). Each trait consisted of five unlinked, biallelic loci, four of which affected the mean of the trait's expression and one which affected its variance. Alleles for loci affecting a trait's mean had an effect size of 1 or -1, and an individual's mean threshold temperature ($\bar{Z}_i$) was offset from the population mean $\bar{Z}$ according to the sum of the allele values:

$$\bar{Z}_i = \bar{Z} + \mu_Z(l_{11} + l_{12} + l_{21} + \ldots + l_{42}) \quad (4)$$

where $l_{jk}$ represents the value attributed to allele k at locus j. Thus, populations were initially seeded with individuals whose allelic composition ($\sum l_{jk}$) could potentially vary from -8 to 8 with a mean of zero; however, $\sum l_{jk}$ could potentially take on any integer value over time due to cumulative mutations (see below). Alleles for the variance locus had an effect size of 1 or 0, and an individual's variance in expression of threshold temperature was calculated as

$$V_{z_i} = \sigma^2_{aZ}(l_{51} + l_{52}) + \sigma^2_{bZ}[2 - (l_{51} + l_{52})], \quad (5)$$

which allowed 1's to represent the value of $\sigma^2_a$ and 0's to represent the value of $\sigma^2_b$. An individual's expressed threshold temperature was then calculated from a normal distribution

of mean $\hat{Z}_i$ with variance $\hat{V}_{zi}$. Individual means and variances for nest-site choice ($N_i$) were also calculated similarly.

Alleles affecting the mean of a trait were allowed to mutate ± 1 value at a rate of $10^{-5}$ per allele per generation (following Bull and Bulmer 1989). Additionally, three null loci affecting the mean of each trait, all originally set to 0 (i.e., initially having no effect on the mean) were allowed to mutate along with the four original alleles concerning a trait's mean, simulating added function and thereby allowing the mean of a trait to evolve outside of its initial phenotypic range if further mutations were acquired at these loci. Ultimately, mutations in this model turned out to be relatively unimportant given the number of generations that simulations were run.

*Model calibration*

The following parameter estimates (± SE) for equation (2) were derived from a multiple linear regression of July air temperatures and nest microhabitat on nest temperatures: intercept ($\hat{\alpha}$): -1.49 ± 2.11; mean July air temperature ($\hat{\beta}$): 1.18 ± 0.09; average percentage of south and west vegetation ($\hat{\gamma}$): -0.035 ± 0.004. No interaction effect between climatic condition and vegetation cover on nest temperatures was present (P = 0.75). The parameters $\alpha$, $\beta$, $\gamma$, and $\varepsilon$ for equation (2) were chosen to be 0, 1.1, - 0.035, and 0, respectively (with a normally distributed variance of 1.0 for $\varepsilon$), based on the derived estimates for $\alpha$, $\beta$, and $\gamma$, and how closely the modeled data fit actual data available from the study population for the ranges and distributions of nest temperatures.

Long-term thermal data reported from a weather station near the field site indicated a normal distribution of July temperatures for the past 50 years ($\bar{x} \pm SD = 23.60 \pm 1.11$ °C; Janzen 1994a). Based on these data, July temperature for each year was thus modeled as a random variable selected from a normal distribution centered around a mean of 23.6 with standard deviation of 1.11.

Adult survivorship varied between 85 and 90% per year for both sexes, which is within the estimates for adult painted turtles in the wild (Wilbur 1975; Mitchell 1988; Zweifel 1989; Iverson and Smith 1993). Survivorship decreased linearly within this range as density of the adult population increased, which kept population sizes stable over time. Offspring mortality rate was calculated arbitrarily as 95.6% through incubation to age 1, which provided a stable population size during constant climatic conditions. This estimate nevertheless falls into clutch and juvenile mortality estimates for several *C. picta* populations [90 - 98%: (Gibbons 1968; Wilbur 1975; Zweifel 1989)].

Distributions of the two traits from the model, N and Z, were compared to actual distributions of nest-site choice and thermal sensitivity of embryonic sex determination from the focal population and published literature. The values $\mu$, $\sigma_a^2$, and $\sigma_b^2$ for threshold temperature (nest-site choice) were chosen to adequately capture known phenotypic ranges for the traits as well as among- and within- clutch (female) phenotypic variation observed for these traits. For threshold temperatures, the combination of these three values determined the range of temperatures that thresholds may vary, as well as *S*, the shape of the transition in sex ratios across temperatures. The values $\mu$, $\sigma_a^2$, and $\sigma_b^2$ set at 0.18, 0.18, and 0.06, respectively, followed levels of among-clutch variation in sex ratio and *S* calculated

from the focal population. When μ, $\sigma_a^2$, and $\sigma_b^2$ were set at 0.10, 0.08, and 0.02, respectively, they followed levels of among-clutch variation in sex ratio and *S* derived from the literature. Table 1 summarizes results from typical simulations of among-clutch variation in sex ratio for clutches across incubation temperatures when $S = -0.38$ and -0.24, respectively. Simulations with $S = -0.24$ are also close to the *S* values calculated for Wisconsin and Tennessee *C. picta* (-0.22 ± 0.0061 and -0.21 ± 0.043, respectively), which are not included in the Table.

The values μ, $\sigma_a^2$, and $\sigma_b^2$ for nest-site choice set at 4, 275, and 125, resepectively, caused the distribution of vegetation cover over nests and repeatability of females to nest microhabitat to closely follow field-collected data. Mean repeatability (± SD) of nest-site choice was 0.23 ± 0.042 from thirty simulations of 180 females laying 3 nests each. This value falls near the range of repeatability estimates calculated for females laying 3 nests each at the field site (0.21; Janzen and Morjan, 2001). Figures 2A and 2B illustrate the distribution of microhabitat for nests in a typical simulation and the distribution for nests in the field, respectively (data from Janzen and Morjan 2001).

Finally, annual offspring sex ratios as a function of yearly climate for a typical simulation are shown in Figure 3. A typical simulation is defined as a simulation exhibiting parameters closest to the mean taken from 20 to 30 simulations. These tables and figures suggest that the model adequately captured biologically relevant levels of variation for these two traits.

*Simulations*

For tests examining relative rates of change in allelic composition of threshold temperatures and nest-site choice, 30 trials simulating 1000 years each were run. In simulations involving climate change, successive changes in the mean temperature of the distribution were adjusted according to the magnitude of total change divided by the transition time (e.g., to simulate a 1-degree increase in temperature over 100 years, the temperature was selected from a normal distribution from which the mean increased 0.01°C per year for 100 years). The variance in climate remained the same during and after the changes. Most simulations involved a 1°C increase or decrease in mean climatic temperature, with the transition period between years 50-150. A 2°C change in this period of time often caused population extinctions.

Changes in threshold temperature and nest-site choice were measured by allelic composition to allow both traits to be compared on the same scale. Allelic composition is simply the sum of the values for all loci concerning the mean expression of a trait in an individual ($\sum l_{jk}$, Eq. 4). Changes in population mean allelic composition for a simulation were calculated by taking the difference in means between the end of the simulation and year 50 (the year immediately before climatic change). Changes in allelic composition (which were normally distributed) for the two traits were compared using paired t-tests.

In some species with TSD, females may conceivably return to nesting grounds as a result of natal imprinting rather than biparental genetic contribution (e.g., Freedberg and Wade 2001). The model was accordingly modified to address this scenario. Using the same initial settings, only the mode of transmission of nest-site choice was changed in the model. In this case, females oviposited in microhabitats with a mean consisting of their own

respective natal microhabitat values, with a maternally inherited variance for nest-site choice as described in Equation 5. All simulations were performed using Borland Delphi 4 (Inprise, Inc.).

*Calculating heritabilities*

Heritabilities ($h^2$) for nest-site choice and nest temperatures were calculated by using mother-daughter regression, and heritability for threshold temperature was calculated by mother-offspring regression, for each of 20 populations of 617 - 956 individuals existing at the end of 50-year simulations of no climate change.

The "effective heritabilities" for both threshold temperatures (effective $h_Z^2$) and nest temperatures (effective $h_T^2$) were calculated by multiplying their respective heritabilities by a relative variance term (Bulmer and Bull 1982; Bull et al. 1982a). The relative variance term accounts for the relative proportion of selection occurring on nest or threshold temperatures through offspring sex ratios, and affects the expected rate of change for each character (Bull et al. 1982a). The effective heritability of threshold temperature is thus:

$$\text{Effective } h_Z^2 = h_Z^2 \frac{\sigma_Z^2}{\sigma_Z^2 + \sigma_T^2} \qquad (4)$$

where $\sigma_Z^2$ is the phenotypic variance for threshold temperature and $\sigma_T^2$ is the phenotypic variance in nest temperatures. Similarly,

$$\text{Effective } h_T^2 = h_T^2 \frac{\sigma_T^2}{\sigma_T^2 + \sigma_Z^2} \qquad (5)$$

Effective heritabilities for nest temperature and threshold temperature are used because offspring sex is affected directly by both factors (Figure 1). Calculating the effective

heritability of nest-site choice on sex ratio is not appropriate because it affects sex indirectly through nest temperature.

Repeatability for nest-site choice in terms of vegetation cover was calculated by first conducting a one-way analysis of variance (ANOVA) and using the within- and among-female mean squares to calculate r (Lessells and Boag 1987, Janzen and Morjan 2001).

## Results

*Calculating S for focal population and from literature*

Both clutch and temperature significantly affected offspring sex ratios for Illinois *C. picta* eggs incubated in the laboratory (Table 1). The shape of the response curve for offspring sex ratio as a function of incubation temperature (*S*, Girondot 1999) for this population was calculated to be -0.45 ± 0.114. The calculated values of *S* for Wisconsin and Tennessee populations of *C. picta* are -0.22 ± 0.0061 and -0.21 ± 0.043, respectively. These values of *S* indicated a significantly narrower transitional range in temperatures producing mixed sex ratios for Wisconsin and Tennessee populations than for the focal population (t-tests: Illinois-Wisconsin: $t_{(4)} = 4.50$, $p < 0.01$; Illinois-Tennessee: $t_{(5)} = 4.45$, $p < 0.001$). Although clutch effects were not reported by Bull et al. (1982b), modelling this narrower transition range of *S* provided similar levels of among-clutch variation in sex ratios to a published study from a Minnesota population of *C. picta* (Rhen and Lang 1998; Table 1). To provide the most conservative comparisons between relative rates of change in threshold temperatures versus nest-site choice, all further results are reported from models using the lower |S| unless indicated otherwise. However, results from simulations with higher |S| are

also shown to demonstrate the evolutionary potential of sex ratios in response to perturbation by climate change.

*Relative rates of evolution for threshold temperature and nest-site choice*

In simulations with no climate changes, adult sex ratios remained stable over time with a mean of 0.5 (Figure 4A). Figure 4B depicts sample simulations plotting mean allelic composition for both traits over time. Higher values for allelic composition are associated with a higher likelihood of producing males, whether through increased vegetation cover causing cooler nests or through higher threshold temperatures. In several simulations, a change in mean allelic composition for threshold temperature was accompanied by a corresponding change in nest-site choice in the opposite direction, due to Fisherian sex-ratio selection. For example, if females nested under increased vegetation cover over time due to genetic drift, threshold temperatures "compensated" by decreasing the likelihood of producing males at cooler nest temperatures. Indeed, a negative correlation for the two traits existed across populations at the end of the simulations ($n = 30$ simulations, $r = -0.72$, $P < 0.0001$). However, no genetic correlation was found for the two traits within individuals ($r$ ranged from 0.1418 to -0.1933 for 100 randomly sampled individuals from each of the 30 simulations, all n.s.). Changes in mean allelic compositions for nest-site choice and mean threshold temperatures did not differ significantly from zero (two-tailed t-test; nest-site choice: $0.040 \pm 1.32$, $t = 0.168$, $df = 29$, $P = 0.87$; threshold temperature: $0.0011 \pm 1.20$, $t = 0.0049$, $df = 29$, $P = 1.0$) or from each other (paired t-test, $t = 0.096$, $df = 29$, $P = 0.92$).

In simulations involving an increase in climate of 1°C over 100 years, adult sex ratios initially became female-biased and then gradually approached 1:1 (Figure 5A). This

return to 1:1 sex ratios is due to Fisherian sex-ratio selection for increased threshold temperatures and for females to place nests under higher vegetation cover, both of which bias offspring sex ratios toward males (Figure 5B). Both threshold temperature and nest-site choice exhibited a change in mean allelic composition significantly greater than zero (two-tailed t-test; nest-site choice ($\overline{N}$): $3.71 \pm 0.91$, $t = 22.25$, $df = 29$, $P < 0.0001$; threshold temperature ($\overline{Z}$): $4.91 \pm 0.73$, $t = 36.75$, $df = 29$, $P < 0.0001$), indicating an increase in the mean for both vegetation cover over nests and threshold temperature. However, threshold temperature experienced a relatively greater change than did nest-site choice (two-tailed paired t-test, $t = 4.74$, $df = 29$, $P < 0.0001$). In other words, there was a greater change in frequency of alleles responsible for producing male-biased clutches than for nesting in cooler sites after a climatic warming. Although all analyses involve $S = -0.24$ because it provides a conservative estimate of relative rate of change in sex ratio compared to nest-site choice, changes in sex ratio over time for $S = -0.38$ are also provided (Figure 5A). In both cases, adult sex ratios remained biased for several hundred years after the change in annual temperatures took place; however, adult sex ratios approached 1:1 more rapidly under higher $|S|$.

Similarly, a decrease in annual temperature of 1°C over 100 years caused sex ratios to become initially male-biased and eventually return to 1:1 (Figure 6A). In this case, several populations went extinct, so a second set of 30 simulations was run and the data combined to analyze the surviving populations. Out of the 60 simulations, 45 populations survived. Both traits exhibited a change in mean allelic composition in the negative direction (two-tailed t-test: nest-site choice: $-3.58 \pm 1.34$, $t = -17.91$, $df = 44$, $P < 0.0001$;

threshold temperature: -4.41 ± 1.15, t = -25.81, df = 44, P < 0.0001; Figure 6B). Although threshold temperature exhibited a relatively greater change than did nest-site choice overall (two-tailed paired t-test, t = 2.53, df = 44, P = 0.015), this relationship was weaker and had greater variability than for simulations of climatic warming (Figure 5B vs. Figure 6B). This is likely due to the populations experiencing a bottleneck during climatic cooling than in simulations of climatic warming (Figure 7). During years of cool temperatures, a scarcity of females in the populations limited annual reproduction and reduced population sizes. Simulations using $S$ = -0.38 exhibited three differences from those using $S$ = -0.24: sex ratios approached 1:1 more rapidly (Figure 6A), threshold temperatures evolved even more rapidly than did nest-site choice (see discussion on heritabilities), and no extinctions occurred during a 1°C cooling in climate (0 of 30 vs. 15 of 60; Fisher's Exact test, $P$ = 0.0018).

Heritabilities for nest-site choice, nest temperatures, and threshold temperatures for the additive genetic model of inheritance are summarized in Table 2. Phenotypic variances (used for calculating effective heritabilities) and effective heritabilities for nest temperatures and threshold temperatures are also included. Although both nest-site choice and threshold temperature exhibited substantial heritabilities, the simulations predicted an extremely low heritability for nest temperature because of the effects of climatic variation on nest temperatures. The simulations also predicted an extremely low effective heritability for threshold temperature because of the relatively large effects of nest temperatures on offspring sex ratios (compare phenotypic variances of nest temperatures vs. threshold temperatures in Table 2).

*Effects of natal imprinting on nest-site choice*

In simulations involving natal imprinting rather than biparental inheritance for nest-site choice with no climatic change, female-biased sex ratios resulted (Figure 8A). Microhabitat selection rapidly and consistently changed to sites with lower vegetation cover (Figure 9A). The greatest change in nest-site choice occurred typically within the first 100 years, and subsequently reached an equilibrium because vegetation cover over nests could not be less than zero. Figure 2C shows the distribution of vegetation cover over nests after 100 years during a typical simulation of natal imprinting to nests with no climate change. Although threshold temperatures subsequently increased to "compensate" for the female-biased sex ratios (Figure 9A), sex ratios even at the end of the 1000-year simulations remained significantly female-biased ($\bar{x} \pm SD = 0.560 \pm 0.75$; hypothesized value = 0.5, $t = 4.37$, $df = 29$, $p < 0.0001$). However, sex ratios slowly but steadily approached 0.50 throughout the simulations, regardless of climate scenario (Figure 8).

Nest-site choice evolved toward microenvironments likely to produce females, regardless of whether the populations experienced an increase or decrease in climate (Figure 9B and 9C). Females oviposited in sites with less vegetation cover, sites which are likely to produce females, even in a climatic warming scenario that produced an excess of females (Figure 9B). The direction of climate change caused relatively small alterations in nest-site choice compared to changes in threshold temperature (Figure 9B and 9C). In contrast to additive genetic inheritance of nest microhabitat, no populations experienced extinction during a 1°C decrease in climatic conditions, likely because sex ratios had already become female-biased before climatic cooling occurred (Figure 9C).

# Discussion

*Evolution of maternal vs. offspring traits affecting sex*

Although many reptilian taxa with TSD exhibit substantial variation in sex ratios among families and populations (Bull et al. 1982a; Bull et al. 1982b; Janzen 1992; Vogt and Flores-Villela 1992; Ewert and Nelson 1994; Rhen and Lang 1998; Bowden 2001), maternal choice of nest sites is generally thought to be more likely to respond than offspring sex ratios to selection (Bull et al., 1982a; Bull et al., 1982b; Bulmer and Bull, 1982). Significant among-clutch variation in sex ratios for laboratory-incubated turtle eggs suggests a high heritability for this trait, but low effective heritabilities are expected in the field (0.03 - 0.06: Bulmer and Bull 1982; Bull et al. 1982a; Janzen 1992). This reasoning is because thermal ranges of natural nests greatly exceed the thermal range where genetic variation in sex ratios is expressed; therefore providing relatively little opportunity for selection to act on this genetic variation in sex ratio. Consequently, variation in thermal choice of nest sites has been thought to be more likely to evolve in the maintenance of sex ratios (Bulmer and Bull 1982; Bull et al. 1982a; Bull et al. 1982b). In contrast, the present study demonstrates that thermal sensitivity of offspring sex is more likely to respond than female nest-site choice to Fisherian selection, given observed levels of variation for these traits in a population of *C. picta* with TSD. However, both traits are likely to have limited evolutionary potential in response to rapid climatic changes in this population, due in part to high variation in nest temperatures attributed to environmental conditions within and across years.

These simulations demonstrate that the effects of biologically relevant levels of among-female variation in nest-site choice on nest temperatures are likely to be masked by

annual fluctuations in climate. Thus its evolutionary potential is reduced to levels even smaller than those estimated for threshold temperatures. These predictions rely on the assumption that there is an additive genetic basis for the inheritance of nest microhabitats and offspring sex ratios. Much of the preceding work concerning the evolutionary potential or dynamics of nest-site choice and threshold temperatures assumed an additive genetic basis for the traits (Bulmer and Bull 1982; Bull et al. 1982a; Janzen 1992; Rhen and Lang 1998; but see Bowden et al. 2000; Freedberg and Wade 2001). Similarly, threshold traits typically have an underlying additive genetic component to them, as well as environmental sensitivity for their expression (reviewed in Roff, 1996). Understanding the basis of the traits' inheritance is critical for evaluating their potential for adaptive responses, as demonstrated by this model: if microhabitats are inherited through natal imprinting, nest-site choice is not expected to evolve adaptively in response to climate change.

In the additive genetic model, nest-site choice evolved more slowly relative to threshold temperature for two main reasons: a low effective heritability for nest temperatures, and the maternal expression of nest-site choice. First, heritability for threshold temperature was higher than the heritability for nest-site choice (Table 2), which may be expected considering the high heritabilities estimated for the former trait in laboratory experiments [$h^2 = 0.82$ for Ouachita map turtles (*Graptemys ouachitensis*): Bull et al. 1982a; $h^2 = 0.26$ - 1 for common snapping turtles (*Chelydra serpentina*): Janzen 1992]. The two studies to date evaluating the upper limit of the heritability of nest-site choice in reptiles with TSD have both estimated values close to 0.20 [leopard geckos (*Eublepharis macularius*): 0.20, Bull et al. 1988; C. picta: 0.21, Janzen and Morjan 2001]. On the surface, nest-site choice appears likely to respond more rapidly to selection because

of its higher heritability than effective heritabilities for offspring sex ratios (~0.20 compared to ~0.06). Although heritability of nest-site choice existed at a significant level in this model (0.22), this value translated into an extremely low heritability for nest temperatures (0.04). Nest temperatures of parents and offspring exhibited little resemblance to each other because of the overriding effect of climatic variation among years. Therefore, females had relatively little control over choosing thermal environments to lay their eggs.

Similarly, Valenzuela and Janzen (2001) did not observe a repeatability in clutch sex ratios for individual female *C. picta* across five nesting seasons, although individual females in this population repeatedly nested in microhabitat qualities correlated with nest temperatures. Additional simulations of my model indicated that the repeatability for individual females in terms of vegetation cover over nests would need to approach 0.60 to evolve at a similar rate as the more conservative estimate ($S$ = -0.22) for threshold temperatures (data not shown). The fact that nest-site choice could evolve at all in these scenarios may be due to the long life-span of the organisms, with annual survivorship rates approaching 90% or greater for painted turtles (Wilbur 1975; Mitchell 1988; Zweifel 1989; Iverson and Smith 1993; but see Frazer et al. 1991). Longer life spans may increase the likelihood that nest temperatures differ among females through microhabitat choice, as climatic fluctuations would tend to "cancel" out over time. In other models, longevity is also considered to enhance selection for or to maintain TSD in reptiles (Bull and Bulmer 1989; Girondot and Pieau 1999).

The second reason that threshold temperature evolved more rapidly than did nest-site choice is because nest-site choice is a maternal effect. The significance of this effect can be demonstrated in my model by allowing nest microhabitat for an egg to be determined by an

offspring's own genotype rather than its mother's (which is unlikely in nature but used to demonstrate this particular point). In this case, nest-site choice evolved more rapidly than did threshold temperatures when climate increased 1°C (two-tailed paired t-test, nest-site choice: $4.76 \pm 1.04$, threshold temperature: $3.88 \pm 0.87$, $t = 2.77$, $df = 29$, $P < 0.001$). Maternal nest-site choice experiences indirect selection because the phenotype on which selection acts directly (sex of the individual) is not expressed by the mother, but by her offspring. These offspring do not carry the genotype for microhabitat selection that affects their sex, but rather a correlated genotype for microhabitat selection through Mendelian inheritance. Since selection acts directly on offspring sex, the evolution of thermal sensitivity of offspring sex depends on the extent that offspring phenotypes and genotypes for this trait are correlated, whereas the evolution of nest-site choice depends on the extent that offspring and maternal genotypes for this trait are correlated. Such dynamics have also been demonstrated to affect epistatic interactions between maternal and offspring traits (Wolf 2000a).

Using mathematical models of polygenic sex determination, Bulmer and Bull (1982) demonstrated that biased sex ratios should return to 1:1 only half as rapidly when under genetic control of the parents than of the offspring. When comparing relative rates of evolution for threshold temperatures and nest temperatures affected by female nest-site choice, Bull et al. (1982a) thus multiplied the effective heritability for nest temperatures by 0.5, because of the female-limited expression of nest-site choice. Since threshold temperatures are expressed by all individuals, and nest-site choice only by females, Bull et al. (1982a) expected that nest-site choice would respond to selection at only half the rate implied by its effective heritability. In my model, the effective heritability for threshold

temperature, as extremely low as it was (0.026, Table 2), remained higher than half the effective heritability for nest temperature (0.0205), which likely explains why nest-site choice evolved more slowly. However, if the reason nest-site choice evolved slowly was due to female-limited expression, we would expect nest-site choice to evolve more rapidly in a female-biased population. Figures 5 and 6 demonstrate that nest-site choice evolved at the same rate regardless of the direction of sex ratio bias, and statistical comparison confirmed that no differences in the magnitude of the change in allelic composition for nest-site choice existed across climatic regimes (t-test: 1°C increase: $3.58 \pm 0.18$, 1°C decrease: $3.72 \pm 0.22$, $n = 75$, $P = 0.65$). These results suggest that indirect selection on nest-site choice, rather than female-limited expression of nest-site choice, is more likely a cause for its inhibited evolutionary response relative to threshold temperatures.

*Effects of natal imprinting*

In sea turtles, mitochondrial haplotypes specific to nesting beaches or even sections of these beaches suggest that females inherit nest sites from their mothers, either genetically or through imprinting (Bowen et al. 1992; Peare and Parker 1996; Bass et al. 1996). Kemp's ridley sea turtle hatchlings were found to orient to sea water that had been in contact with incubation substrate, suggesting a natal imprinting mechanism for inheritance of nesting beaches (Grassman et al. 1984). In populations exhibiting female nest-site philopatry, nesting frequency is expected to increase in sites likely to produce females, causing a runaway process resulting in female-biased sex ratios (Bull 1980; Reinhold 1998; Freedberg and Wade 2001). This phenomenon is expected to occur because female-producing sites are the sites to which many individuals return to nest in subsequent generations, whereas sites

producing mostly males produce few females that return to nest. My model confirms that female-biased sex ratios may arise under philopatry without climate change. Most importantly, it shows that nest-site choice responded minimally during climate change scenarios and in fact was maladaptive during climatic warming, with females laying eggs in female-producing environments.

Long-term study of the focal population of *C. picta* on which this model is based indicates that females repeatedly return to nest at this beach and also nest repeatedly at particular microhabitats within this beach (Valenzuela and Janzen 2001). Although nest-site fidelity to specific geographic areas occurs within females, it occurs at a scale that is too large to explain repeatability in overstory vegetation cover over nests (Janzen and Morjan 2001; Valenzuela and Janzen 2001). Females repeatedly nest within specific geographic areas, but repeatability of nesting to specific microhabitats is not due to geographic location because these areas contain a high level of microhabitat heterogeneity within them. Although there are insufficient data to support or refute a genetically-inherited vs. imprinting scenario to nest sites in this population, my simulations predict that in the case of natal imprinting to nest microhabitats, (1) female-biased adult sex ratios should occur, and (2) the distribution of vegetation cover over nests should favor female-producing sites. Annual offspring sex ratios combined with adult capture records suggest an even or possibly male-biased sex ratio in this population (Janzen 1994a; F. J. Janzen, pers. comm.). If imprinting of females to particular microhabitats is responsible for the observed repeatability for this behavioral pattern, the current distribution of vegetation cover over nests should change rapidly over a relatively short time span in this population of painted turtles, with most nests being laid under no vegetation cover (Figure 2C). The most rapid rate of change

is expected to occur during the initial years (Figure 9A), along with a rapid change toward female-biased sex ratios (Figure 8A). The observations that the distribution of vegetation cover over nests has favored intermediate sites for the past 13 years of study (2 to 3 generations), and female-biased sex ratios are not evident in this population, suggest against a possible mechanism of natal imprinting to nest microhabitats that affect sex ratios in this population.

Under scenarios with no climate change, natal imprinting should also select against TSD because higher threshold temperatures producing male-biased clutches are favored (Figure 9A). Freedberg and Wade (2001) suggest that alleles responsible for decreasing the precision of philopatry are unlikely to invade a population, because of the fitness advantage females acquire by returning to sites proven to produce surviving offspring. If natal imprinting occurs in this focal population of *C. picta*, it may occur at a geographic (but not microhabitat-related) scale, which could potentially allow continual use of general sites that produce surviving offspring without skewing sex ratios. Female-biased sex ratios should only occur where sufficient thermal variation exists across habitats inherited through imprinting to differentially affect sex ratios. This scenario could resolve why female-biased sex ratios are thought to occur in sea turtles (reviewed in Freedberg and Wade 2001), where thermal variation is likely to be greater across, instead of within, nesting beaches (e.g., Hays et al. 2001), but inconsistently in freshwater turtles and crocodilians with TSD (Gibbons 1990; Thorbjarnarson 1997; Lance et al. 2000).

*Implications for variation in natural populations*

In addition to nest-site choice, propagule size (Roosenburg 1996; Roosenburg and Niewiarowski 1998) and provisioning of yolk steroids (Janzen et al. 1998; Bowden et al. 2000) may also play important roles in the maintenance of TSD or for sex ratio evolution in this sex determining system. Recent developments in evolutionary theory underscore the importance of understanding the role of maternal effects in evolutionary processes. For example, the development of multiple fitness peaks through epistatic interactions of maternal and offspring genotypes has been suggested to result in evolutionary dynamics varying from runaway processes to impaired responses to selection (Wade 1998; Wolf 2000a). Such theoretical predictions of responses of maternal effects (indirect genetic effects) vs. direct genetic effects on offspring traits to selection provide the framework for further evaluating gene-culture interactions (e.g., threshold temperatures with natal philopatry; Freedberg and Wade 2001), and the evolutionary dynamics of natural populations (reviewed in Wolf et al. 1998).

Phenotypic variation in sex ratios observed across clutches was assumed to have an additive genetic basis in my model. Maternal effects and dominance variance may also affect among-family phenotypic variance in these traits (Falconer and Mackay 1996). Although evaluations of the evolutionary potential for among-clutch variation in sex ratios have assumed no maternal effects (Bull et al. 1982a; Janzen 1992; Rhen and Lang 1998), recent evidence suggests that clutch sex ratios are affected by yolk steroid hormone concentrations (Janzen et al. 1998) that vary seasonally (Bowden et al. 2000). Although the basis for a seasonal increase in yolk estradiol concentrations is currently unknown, this variation is likely to be environmentally induced (e.g., seasonally-induced fluctuations in levels of circulating steroids in females; Janzen et al., unpubl. data). Sex ratio variation

across clutches may be enhanced by this maternal component, possibly reducing heritability estimates. For this reason, my model may provide an upper estimate of the evolutionary potential for sex ratios in this population, which is nevertheless low.

On the other hand, the model may provide a lower estimate of the evolutionary potential for offspring sex ratios if genotype-by-environment interactions exist in *C. picta*. Rhen and Lang (1998) suggested that genotype-by-environment interactions increase the evolutionary potential of sex ratio in this system by allowing changes in the shape of the response curve of sex ratio to temperature, rather than simply shifting the response curve to the left or right, in response to climate change. Although the transition range producing mixed sex ratios is wider in the Illinois population than in two other populations studied, no genotype-by-environment interactions for clutch sex ratios have yet been found within populations of *C. picta* (Rhen and Lang 1998; Table 1). However, in my study, eggs would likely need to be incubated at fewer than 5 temperatures to detect such an interaction since clutch sizes were small (12 eggs per clutch on average in this population).

If selection acts on offspring sex ratios, for sex ratio evolution across varying climatic regimes, a cline wherein lower pivotal temperatures occur in higher latitudes might be expected. In reality, latitudinal clines in pivotal temperatures for turtles have been opposite to the direction expected, if any cline is found at all (Bull et al. 1982b; Mrosovsky 1988; Vogt and Flores-Villela 1992; Ewert et al. 1994). Although geographic variation in nest-site choice among populations was described as a possible explanation for this trend (Bull et al. 1982b; Ewert et al. 1994), this has remained largely uninvestigated in reptiles with TSD. My model also predicts that thermal sensitivity of offspring sex ratios is likely to coevolve with patterns of genetically inherited nest-site choice under stable climatic

conditions (Figure 4B). When making geographic comparisons, it would be critical to compare actual nest temperatures across latitudes to determine if developing embryos in fact experience different thermal environments (e.g., Morjan 2002).

One major assumption and potential caveat of this model is that nesting dates were assumed to be fixed in the population. Changes in nesting dates could mitigate effects of climatic changes on nest temperatures; perhaps females could nest earlier in the season during hotter years. Nesting phenology changes with climatic condition in some populations of birds (e.g., Dunn and Winkler 2000), but remains undocumented in reptiles except for some casual observations. This assumption of fixed nesting dates in part follows implicit assumptions in past studies for the evolution of sex ratios through maternal behavior (Bull et al. 1982a; Bulmer and Bull 1982; Janzen 1992; Ewert et al. 1994; but see Bull et al. 1982b; Vogt and Bull 1982). Since differences in nesting dates between northern and southern populations of three species of freshwater turtles were thought to mitigate climatic differences on nest temperatures only minimally, Bull et al. (1982b) suggested that maternal choice of nesting environments was likely important. However, the main reason nesting dates remained fixed in this model because such variation lacks empirical evidence in *C. picta bellii* both within the Illinois population (Janzen 1994a) and across their range (Christiansen and Moll 1973; Morjan 2002). Illinois *C. picta* females nest during the month of June regardless of whether July is relatively warm or cool (Janzen 1994a). No repeatability of individual females for nesting dates or relative order of nesting has been found for females in this population (F. Janzen and C. Morjan, unpubl. data). *Chrysemys p. bellii* in New Mexico, which typically experience July temperatures >2°C higher than their Illinois conspecifics, also nest at almost exactly the same time as the Illinois population

(Morjan 2002). Latitudinal trends in nesting phenology may be present in other North American freshwater turtles (Moll 1979), but further investigation is needed to make strong conclusions for other chelonian taxa.

Chevalier et al. (1999) report a French Guiana population of leatherback sea turtles (*Dermochelys coriacea*) exhibiting a narrower range for $S$ than a population from Costa Rica, and speculated that a small $|S|$ could reflect a low level of genetic polymorphism for sex ratio in the French Guiana population. In my model, differences in $S$ that reflect phenotypic differences among populations of *C. picta* determined population extinction versus survival during scenarios of climatic cooling. Although heritabilities were similar for both variations of $S$, it was the phenotypic range of threshold temperatures that allowed more rapid response to selection (Table 2). The differences in $S$ detected between the Illinois *C. picta* population and those from Wisconsin and Tennessee is unlikely to be explained by the smaller number of clutches used in the Illinois study because $|S|$ was larger for Illinois. Smaller sample sizes would have been less likely to detect variation in sex ratios at the ends of this transition range, producing an artifactually smaller $|S|$ instead.

*Evolutionary potential of sex ratio*

In the near future, global climatic temperatures are expected to increase due to human activities by 1-5°C per 100 years (Schneider 1989; Root and Schneider 1993; O'Neill et al. 2001). Recently derived models predict an increase of about 1.5°C within the next 50 years in North America (Zwiers, 2002). Rates of climatic changes at the lower end of this range were modeled because many simulations involving greater changes resulted in high

levels of extinction. For example, a 2°C change in climate in either direction over 100 or even 200 years caused most populations to become extinct, even while optimistically assuming that threshold temperatures and nest-site choice can evolve through additive genetic inheritance. Extinction rates under these scenarios, however, were found to be exquisitely sensitive to even small changes in survivorship in most life stages, as well as to density-dependence functions, *S*, and population size. Nevertheless, the results for relative rates of evolution of nest-site choice and threshold temperatures remained robust to changes in these factors. Consequently, the implications for this model mainly concern the relative rates of evolution for maternal and offspring traits affecting sex ratios; more data are required to accurately model predicted effects of climate change on population dynamics or extinction rates. My model also does not include Allee effects of mate search strategies (Berec et al. 2001) or metapopulation dynamics involving various nesting beaches with different microhabitat-temperature functions, both of which may also be critical for projections of extinction risk.

Adaptive mechanisms potentially counteracting Fisherian sex ratio selection in reptiles have received little consistent empirical support (reviewed in Shine 1999). For example, group-structured adaptations or adaptively biasing clutch sex ratios to avoid inbreeding among siblings are inconsistent with life history traits of reptiles with TSD (Ewert and Nelson, 1991; Burke, 1993; Shine, 1999). Although TSD as an adaptive mechanism to allocate offspring sex to that which is best suited for its incubation environment (Charnov and Bull 1977) has been clearly demonstrated for silverside fishes (*Menidia menidia*) (Conover 1984), this explanation lacks widespread and consistent empirical support concerning reptiles (reviewed in Shine, 1999). Fisherian sex ratio

selection has been demonstrated in silverside fishes that exhibit ESD in addition to GSD: biased sex ratios in laboratory-raised populations kept under constant thermal conditions were readily restored to 1:1 through only 6 to 8 generations of selection (Conover and Van Voorhees 1990). Biased sex ratios were also restored to 1:1 through sex ratio selection in *Drosophila* hybrids within 16 generations (Blows et al. 2001). On the other hand, Fisherian selection was observed to act quite slowly (expected to take 330 generations) in an experimental population of *Drosophila mediopunctata*, and was expected to evolve slowly in general (Carvalho et al. 1998). Variation for maternal and offspring traits affecting sex ratio in this population of *C. picta* suggests that sex ratios in reptiles with TSD should evolve slowly in response to expected climate changes. However, one important aspect of this expected rate is a relatively long generation time for turtles. Although the simulation model required about 550 years to restore sex ratios after climatic warming (Figure 5), this may be about 50 - 100 generations on average for turtles. Therefore, in terms of generation time, expected rates of evolution may fall within the ranges of empirical studies in other organisms. Nevertheless, projected climate changes may be quite rapid compared to the expected time required to restore sex ratios in turtles.

Interactions between maternal and offspring traits are common in nature, such as decisions in host choice for ovipositing females, mode of seed dispersal, and propagule size (reviewed in Mousseau and Fox 1998a). In my model of TSD, a trait affecting offspring sex that was expressed by the offspring (threshold temperature) responded more readily to restore biased sex ratios than a trait expressed maternally (nest-site choice), regardless of whether nest-site choice was inherited through genetic preference or imprinting to sites. These results seem paradoxical in the sense that variation across nest microenvironments

within a year varied on average by 6.4°C due to variation in nest-site selection by females, whereas threshold temperatures varied by only half this amount, at best. Previous theory based on similar evaluations of phenotypic variation for the two traits concluded that maternal choice of oviposition environment may be likely to respond more readily to selection (Bull et al. 1982a). However, evolutionary dynamics of maternal effects may include nonintuitive responses such as time-lags, temporary maladaptive responses to selection, or runaway processes (Kirkpatrick and Lande 1989; Wade 1998). Furthermore, it is important to quantify the amount that environmental variation modifies maternal effects (reviewed in Rossiter 1998), as demonstrated by effects of yearly climate in this scenario. Besides modifying their nesting behavior or threshold temperatures, reptiles with TSD are thought to face three alternative responses to climate change: modify their geographic ranges, change to genotypic sex determination, or face extinction (Janzen and Paukstis 1991a). In light of the predicted outcomes by my simulation model, maternal selection for nest microhabitats in these reptiles may be relatively ineffective in response to sex-ratio perturbation caused by rapid climate change.

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Zwiers, F. W. 2002. The 20-year forecast. Nature 416:690-691.

**Table 1.** Offspring sex as a function of temperature from 12 clutches of Chrysemys picta eggs from Thomson, Illinois (n = 138), from typical simulations of two *S* parameter values (8 eggs per simulated clutch). Likelihood ratio $\chi^2$ values from logistic regression are provided. Data from *Chrysemys picta* (Rhen and Lang, 1998) are provided for comparison.

| Experiment | N | *S* | Effect | LR$\chi^2$ | df | P |
|---|---|---|---|---|---|---|
| Illinois | 138 | -0.45 ± 0.11 | Clutch | 29.28 | 11 | 0.0021 |
| | | | Temperature | 79.80 | 4 | < 0.0001 |
| | | | Clutch X Temperature | 39.05 | 44 | 0.68 |
| Typical | 144 | -0.38 ± 0.06 | Clutch | 26.64 | 11 | 0.0052 |
| simulation | | | Temperature | 89.31 | 4 | < 0.0001 |
| | | | Clutch X Temperature | 32.66 | 44 | 0.89 |
| Typical | 288 | -0.24 ± 0.27 | Clutch | 52.5 | 23 | 0.0004 |
| simulation | | | Temperature | 143.3 | 2 | <0.0001 |
| | | | Clutch X Temperature | 44.9 | 46 | 0.52 |
| Rhen and | 268 | N/A | Clutch | 53.7 | 23 | 0.0003 |
| Lang (1998) | | | Temperature | 152.7 | 2 | <0.0001 |
| | | | Clutch X Temperature | 23.7 | 46 | 0.98 |

**Table 2.** Heritabilities ($h^2$), phenotypic variances (used to calculate effective heritabilities), and effective heritabilities for nest-site choice, nest temperatures, and threshold temperatures for 20 simulations of 617 - 956 parent-offspring pairs at the end of 50-years.

| Trait | $h^2 \pm SD$ | Phenotypic variance | Effective $h^2$ |
|---|---|---|---|
| nest-site choice | 0.22 ± 0.086 | - | - |
| nest temperature | 0.044 ± 0.20 | 2.59 | 0.041 |
| threshold temperature ($S$ = -0.24) | 0.44 ± 0.089 | 0.17 | 0.026 |
| threshold temperature ($S$ = -0.38) | 0.50 ± 0.058 | 0.45 | 0.074 |

**Figure 1.** Simulated life cycle of *Chrysemys picta*.

**Figure 2.** Distributions of percentage of vegetation cover over nests ($\bar{x} \pm SD$) from (A) a typical 3- year simulation (N = 540) (B) for all *Chrysemys picta* nests from Thomson, Illinois from 1995-1999 (N = 887), and (C) after 100 years during a typical simulation involving natal imprinting to nest microhabitats.

**Figure 3.** Results from a typical simulation of annual offspring sex ratio as a function of yearly climatic condition. The linear regression of annual cohort sex ratio (% male) as a function of mean July air temperature reported for this population of *Chrysemys picta* is Y = -25.59 (mean July air temperature) + 651.25, r = 0.91, P = 0.03 (Janzen, 1994a).

**Figure 4.** Results from sample simulations of *Chrysemys picta* populations without climatic change. (A) Mean population sex ratios. Standard errors for the mean were +/- 1 percent. (B) Examples of temporal changes in mean allelic composition in the populations for threshold temperature and vegetation cover over nests. Increasing values of allelic composition indicates an increase in the population mean for that trait. In the center and right panels, the traits diverged through genetic drift.

**Figure 5.** (A) Mean sex ratios by year for a 1°C increase in climate. Black lines represent mean annual temperature. Dark gray and light gray lines represent adult sex ratio when S = 0.24 and S = 0.38, respectively. Standard errors for sex ratio at $S = 0.24$, $S = 0.38$ are +/- 1.0 and 0.015 percent, respectively, and for annual temperature, +/- 0.20°C. (B) Temporal

changes in mean allelic composition in the populations for threshold temperature when $S$ = 0.24 (solid black line) and vegetation cover over nests (solid gray line). Standard errors are represented by dotted lines.

**Figure 6.** (A) Mean sex ratios by year for a 1°C decrease in climate. Black lines represent mean annual temperature. Dark gray and light gray lines represent adult sex ratio when S = 0.24 and S = 0.38, respectively. Standard errors for sex ratio at S = 0.24, S = 0.38 are +/- 1.0 and 0.015 percent, respectively, and for annual temperature, +/- 0.16°C. (B) Temporal changes in mean allelic composition in the populations for threshold temperature when $S$ = 0.24 (solid black line) and vegetation cover over nests (solid gray line). Standard errors are represented by dotted lines.

**Figure 7.** Adult population size over time in typical simulations of a climatic warming (A) or cooling (B).

**Figure 8.** Adult sex ratios for natal imprinting to nests with similar microhabitats as birth sites during (A) no climatic change, (B) a 1 °C increase in climate, and (C) a 1 °C decrease in climate.

**Figure 9.** Changes in mean allelic composition for threshold temperatures (black solid lines) and oviposition-site choice (gray solid lines) for natal imprinting to nests during (A) no climate change, (B) a 1 °C increase in climate, and (C) a 1 °C decrease in climate. Standard errors are represented as dashed lines.

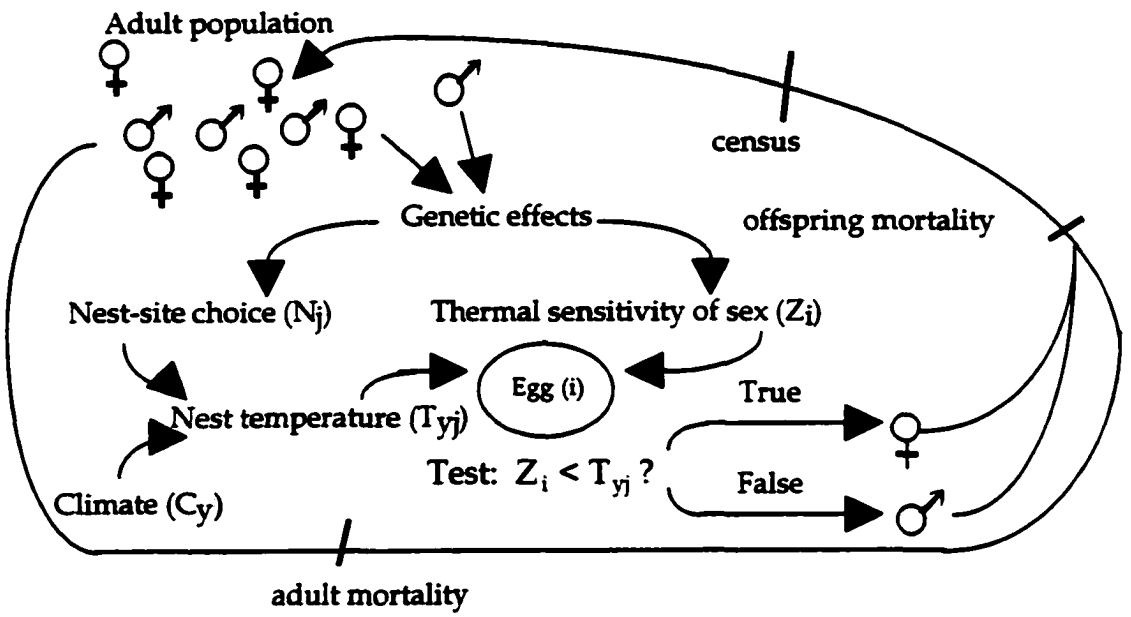
Adult population
census
Genetic effects
offspring mortality
Nest-site choice (Nj)
Thermal sensitivity of sex (Zi)
Egg (i)
True
Nest temperature (Tyj)
Test: Zi < Tyj ?
False
Climate (Cy)
adult mortality

**Figure 1.**

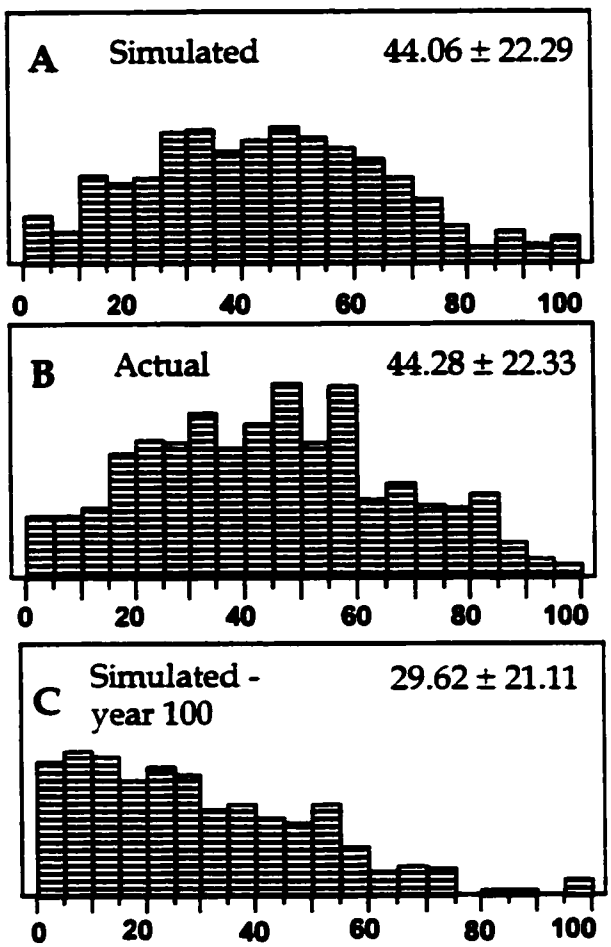
A
Simulated
44.06 ± 22.29
0
20
40
60
80
100
B
Actual
44.28 ± 22.33
0
20
40
60
80
100
C
Simulated -
year 100
29.62 ± 21.11
0
20
40
60
80
100
% vegetation cover over nests

**Figure 2.**

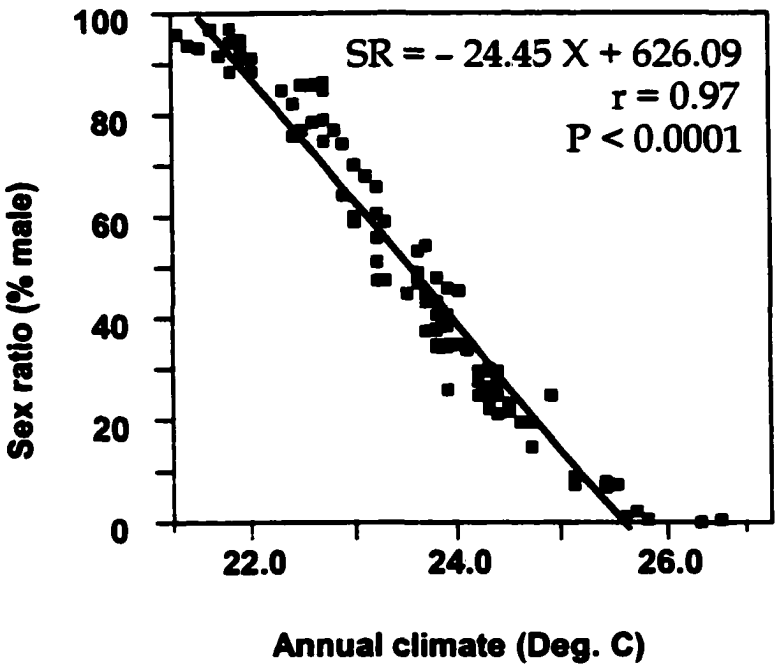
SR = - 24.45 X + 626.09
r = 0.97
P < 0.0001
Sex ratio (% male)
100
80
60
40
20
0
22.0
24.0
26.0
Annual climate (Deg. C)

**Figure 3.**

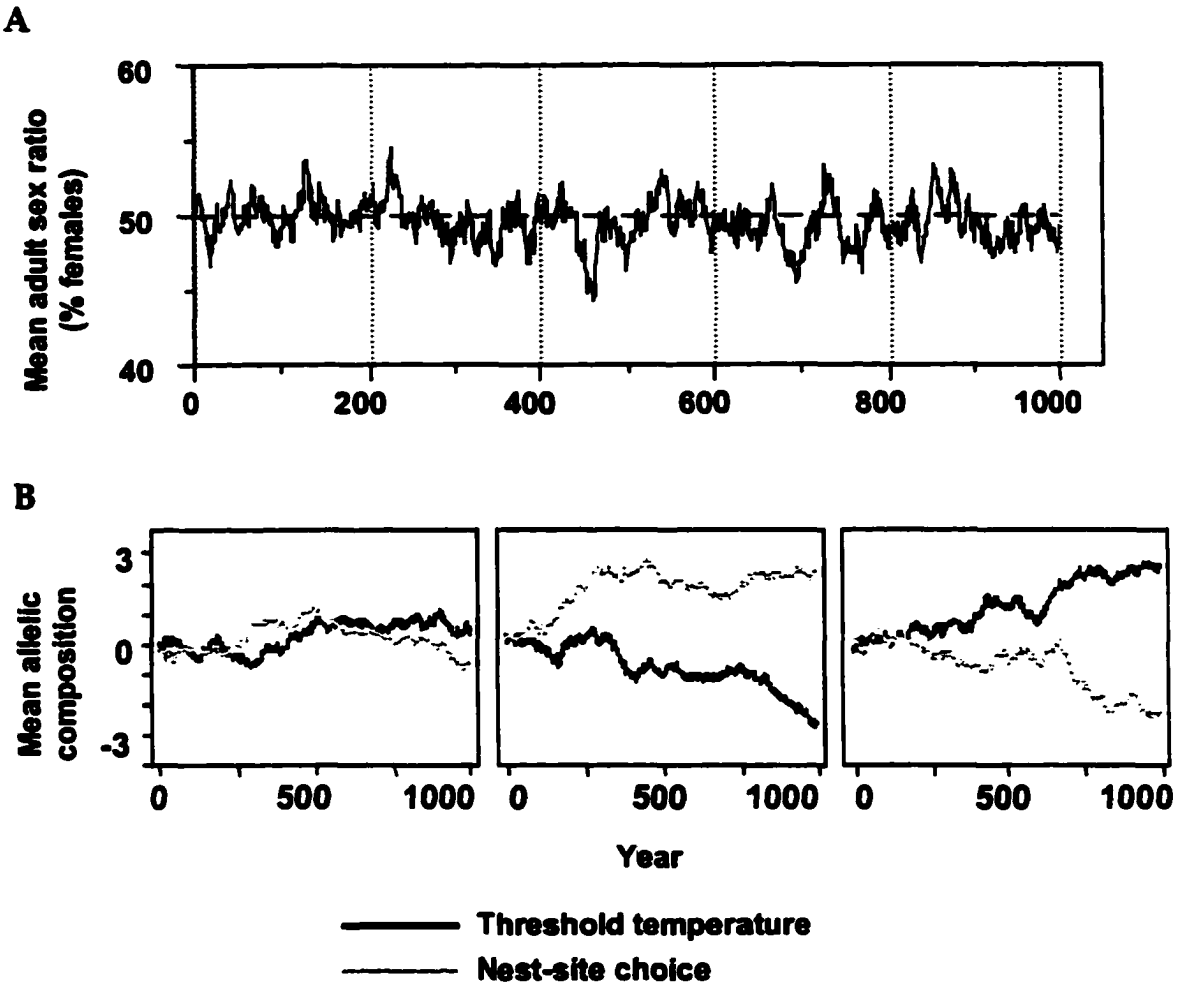
A
Mean adult sex ratio (% females)
60
50
40
0
200
400
600
800
1000
B
Mean allelic composition
3
0
-3
0
500
1000
Year
Threshold temperature
Nest-site choice

**Figure 4.**

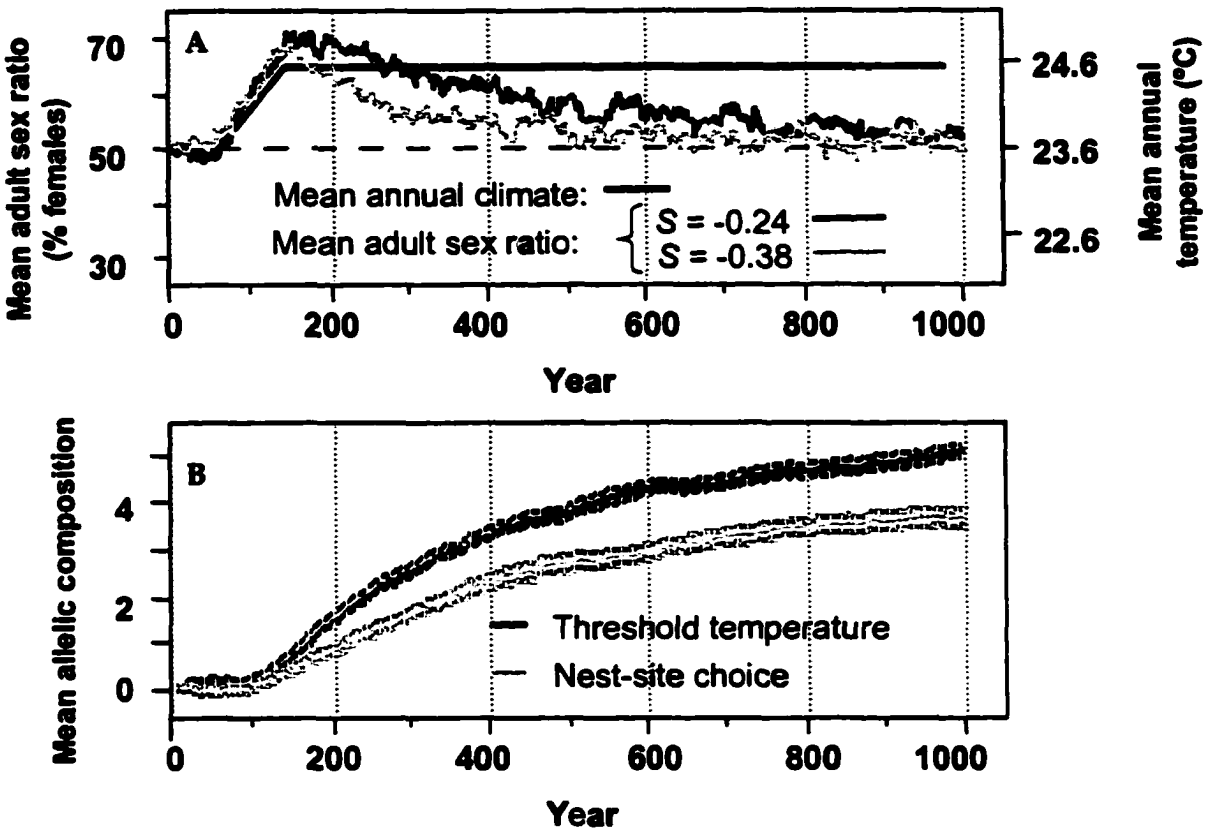
A
Mean adult sex ratio (% females)
70
50
30
Mean annual climate:
Mean adult sex ratio:
S = -0.24
S = -0.38
24.6
23.6
22.6
Mean annual temperature (°C)
0
200
400
600
800
1000
Year
B
Mean allelic composition
4
2
0
Threshold temperature
Nest-site choice
0
200
400
600
800
1000
Year

**Figure 5.**

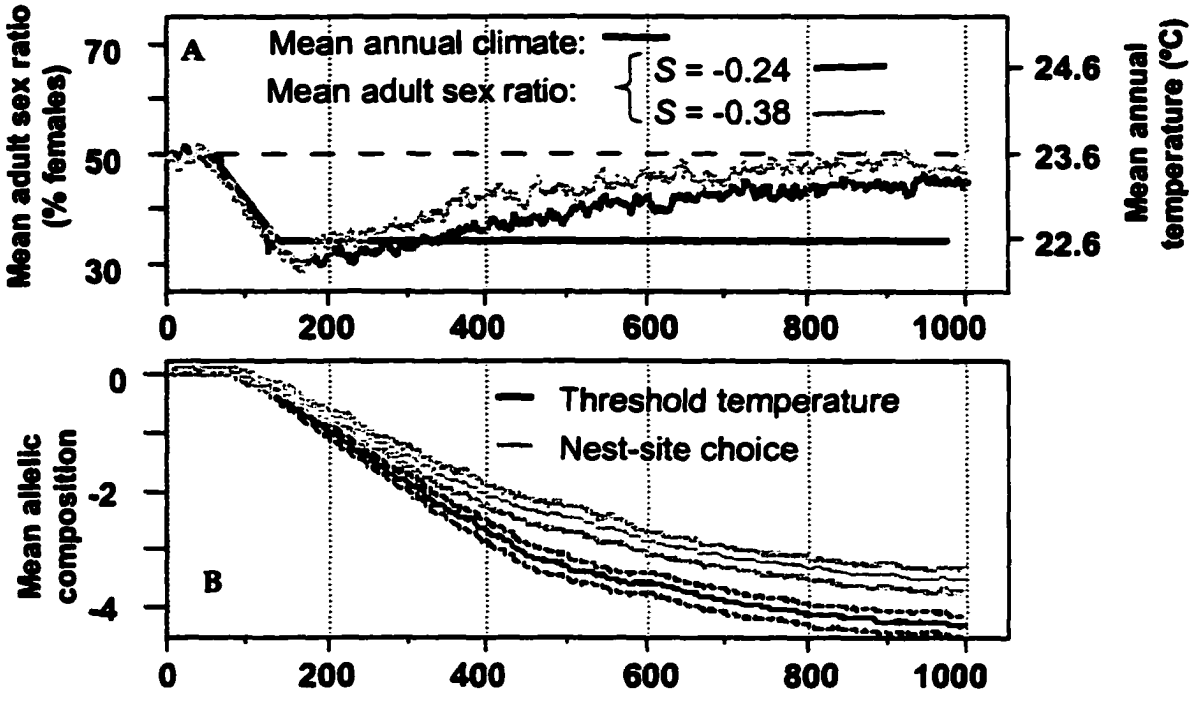
Mean adult sex ratio (% females)
70
50
30
A
Mean annual climate:
Mean adult sex ratio:
S = -0.24
S = -0.38
24.6
23.6
22.6
Mean annual temperature (°C)
0
200
400
600
800
1000
Mean allelic composition
0
-2
-4
B
Threshold temperature
Nest-site choice
0
200
400
600
800
1000

**Figure 6.**

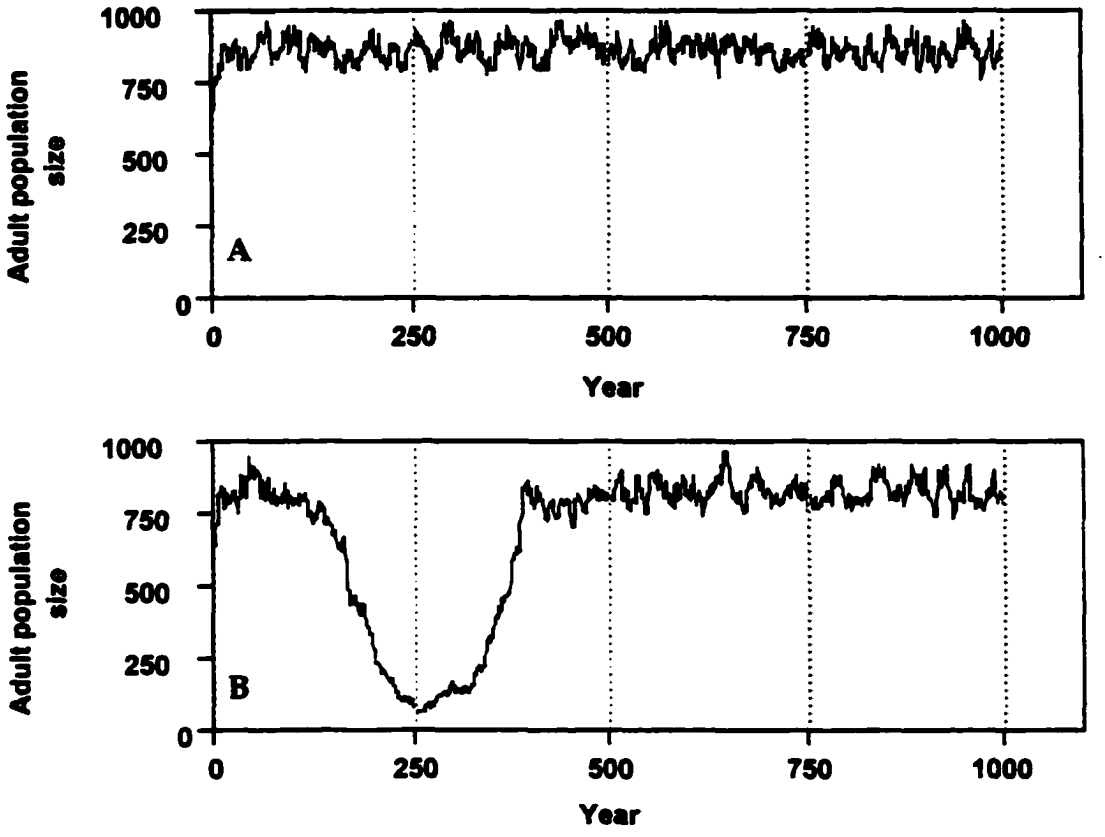
Adult population size
1000
750
500
250
0
A
0
250
500
750
1000
Year
Adult population size
1000
750
500
250
0
B
0
250
500
750
1000
Year

**Figure 7.**

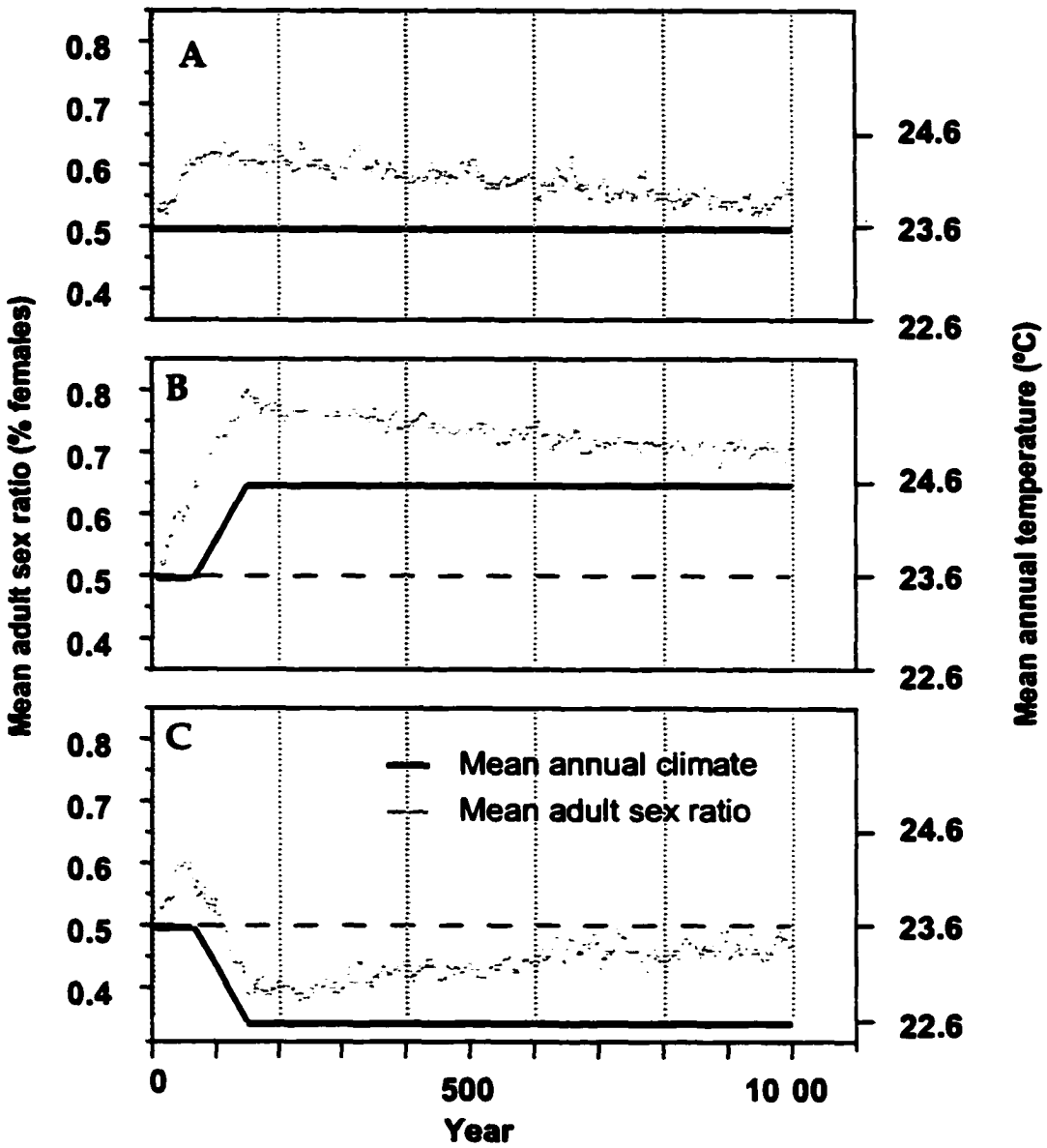
A
B
C
0.8
0.7
0.6
0.5
0.4
24.6
23.6
22.6
Mean adult sex ratio (% females)
Mean annual temperature (°C)
Mean annual climate
Mean adult sex ratio
0
500
10 00
Year

**Figure 8.**

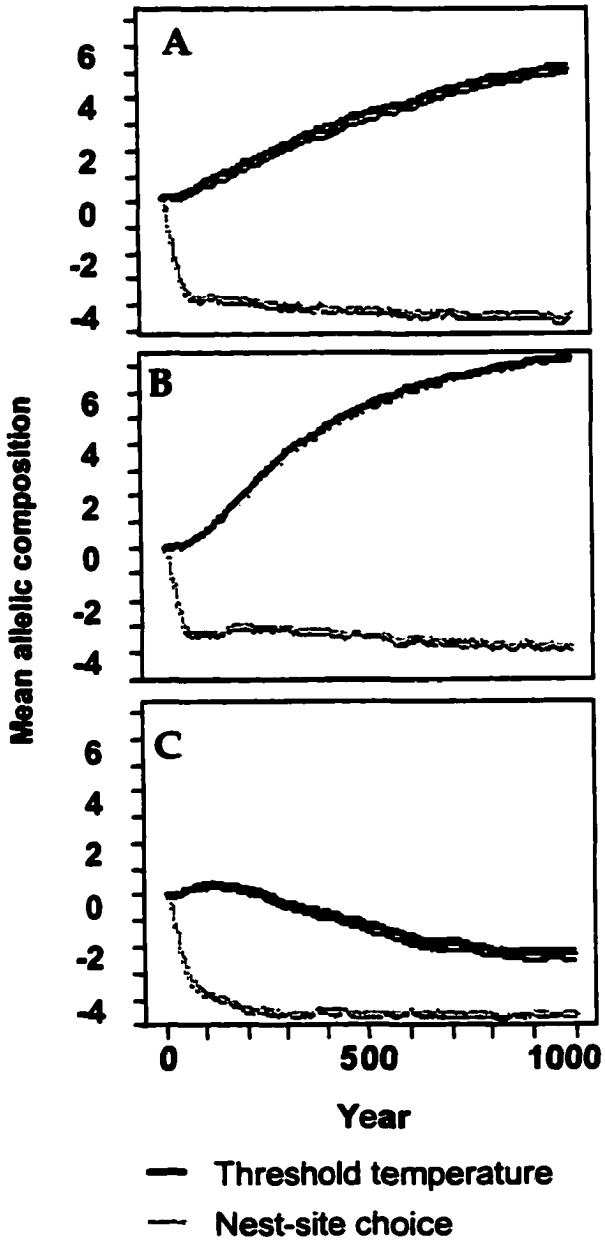
A
B
C
6
4
2
0
-2
-4
Mean allelic composition
0
500
1000
Year
Threshold temperature
Nest-site choice

**Figure 9.**

# CHAPTER 6.

## Evolution of environmental sex determination from genotypic sex determination: is a differential fitness function necessary?

A paper to be submitted to Evolution

Carrie L. Morjan

Department of Zoology and Genetics, Iowa State University

Ames, IA 50011

## ABSTRACT

Despite the discovery of environmental sex determination (ESD) in reptiles almost 40 years ago, its proximate mechanisms and adaptive significance remain poorly understood. The most widely accepted hypothesis for its adaptive significance proposes that environmental incubation conditions provide a higher fitness for one sex in some patches, and for the other sex in other patches. However, little consistent or convincing data demonstrate such a differential fitness effect in reptiles with temperature-dependent sex determination (TSD, a form of ESD). Through computer simulation models, I demonstrate that TSD may invade a population exhibiting genotypic sex determination (GSD) for individuals experiencing a variable incubation environment, without including the differential fitness condition mapping optimal thermal conditions to sex. TSD may invade GSD as a relatively neutral strategy, or with an advantage over GSD by forming individuals homozygous for major sex determining genes with reduced fitness. Furthermore, natal philopatry to incubation sites may enhance the invasion success of TSD in a GSD system, but does not effectively eliminate both sex chromosomes. Although the lability of GSD and

TSD across reptilian taxa has been suggested to support the adaptive significance of TSD, it may instead suggest a more or less neutral status for either sex determining system.

## Introduction

In genotypic sex determination (GSD), sex is determined at conception by Mendelian segregation of the sex chromosomes. However, in environmental sex determination (ESD), sex is determined by environmental effects on the embryo some time after fertilization. Why some organisms leave sex determination to the vagaries of the environment is a question prompting much speculation, especially as ESD is considered maladaptive compared to GSD through Fisherian sex ratio selection (Bull, 1983). Consequently, ESD has widely been considered an adaptation to particular life history traits (Charnov and Bull, 1977; Bull, 1983; reviewed in Shine, 1999). Although an adaptive explanation for ESD seems likely for some invertebrates and a fish (Charnov and Bull, 1977; Bull, 1983; Conover, 1984), the presence and persistence of temperature-dependent sex determination (TSD, a form of ESD) in reptiles remains an enigma (reviewed in Shine, 1999).

Three major hypotheses address potential adaptive functions of TSD in reptiles (Bull, 1983; Ewert and Nelson, 1991; Burke, 1993; Shine, 1999). Burke (1993) and Shine (1999) pointed out discrepancies between two of these adaptive hypotheses and basic life history traits of reptiles, highlighting the third as the most logically sound. This third hypothesis follows a fundamental condition that ESD would be adaptive primarily when fitness for one sex is optimal in some incubation conditions, and for the other sex in other incubation conditions (Charnov and Bull, 1977). GSD would then experience a

disadvantage to ESD because an embryo with GSD is assigned sex prior to entering its environment and hence is more likely to be the sex of lower fitness. Yet major difficulties with the differential fitness condition of the Charnov-Bull model have not been adequately resolved. For example, most reptiles with TSD are long-lived and may take several years to reach reproductive maturity (Janzen and Paukstis, 1991a; Girondot and Pieau, 1999). How relatively minor differences in incubation temperature affect lifetime reproductive success among individuals, independent of various other factors (e.g., nutrition) affecting development and growth, remain undocumented (Shine, 1999). Furthermore, since patterns of TSD in reptiles do not vary consistently with temperature (i.e., hot temperatures produce females in most turtles but produce males in lizards), separate scenarios matching differential fitness benefits to different life history traits are necessary for each pattern of TSD. Finally, defining what the actual fitness benefit is for each sex leads to a long list of alternatives to be measured (e.g., growth rates, antipredator behavior, mate choice, etc.) in lieu of measuring lifetime reproductive success. This problem makes it difficult to ever confidently reject the Charnov-Bull model (see Brown, 2001, for this argument concerning the Trivers and Willard hypothesis).

On the other hand, TSD may not have any adaptive significance and simply persists as an ancestral trait (Janzen and Paukstis, 1988; Ewert and Nelson, 1991; Burke, 1993; Shine, 1999). It is unclear whether GSD or TSD is ancestral in reptiles (Janzen and Paukstis, 1991a; Burke, 1993). TSD is present in tuataras (Cree et al., 1995), crocodilians, in several lizards, and in most turtles, but has not been found in snakes (reviewed in Janzen and Paukstis, 1991a). Amphibians generally exhibit GSD (Hayes, 1998), but extreme temperatures will reverse sex in some species (Bull, 1980). Even if ESD is assumed to be

ancestral in amniote vertebrates, it likely evolved from GSD at least twice in lizards (Janzen and Paukstis, 1991b). Regardless, some plausible mechanism for ESD to arise from GSD is necessary to seriously consider a possible derived status of TSD in reptiles. The only such model is provided by Bull (1981). Bull (1981) first proposes that all homogametic individuals (e.g., XX) must have a tendency to reverse sex under extreme environmental conditions. Secondly, there must be pre-existing, heritable genetic variation for this tendency to reverse sex. Finally, for ESD to be favored over GSD, an adaptive function such as the Charnov-Bull model must be included. Although this model seems realistic and is a good start, the differential fitness condition generally lacks empirical support in reptiles. Nevertheless, little progress has been made during the past 20 years in developing alternative mechanisms for the origin or maintenance of ESD without incorporating some form of this "differential fitness" component (Shine, 1999). Given the disturbing fact that the only convincing data demonstrating such a differential fitness benefit for TSD occur in a fish (Conover, 1984), the possibility of its absence in TSD (or the absence of an adaptive value for TSD in general) deserves more attention.

ESD in fact may be relatively neutral compared to GSD when a large number of overlapping generations participate in reproduction (Girondot and Pieau, 1996; 1999). In long-lived, iteroparous species, the potential reproductive success of an individual is not related to the primary sex ratio of its own cohort, but to the weighted average of the primary sex ratios of all the cohorts participating in reproduction (Girondot and Pieau, 1999). The large number of participating cohorts in a long-lived species would then buffer the effect of temporal environmental variation on primary sex ratios among years. Therefore, a GSD individual gains little benefit in a population with ESD that exhibits only minor fluctuations

in this average primary sex ratio. Indeed, Bull and Bulmer (1989) mathematically demonstrated that long-lived species experience weaker selection against TSD than do short-lived species. In reptiles with long life spans and a large number of overlapping generations, perhaps ESD could evolve and be maintained in fairly significant frequencies as a relatively neutral strategy. These hypotheses for a relatively neutral role of TSD (Janzen and Paukstis, 1988; Girondot and Pieau, 1999) assume that TSD may persist because it is generally not maladaptive; however, they do not address the possibility that TSD could be derived out of GSD but have no adaptive significance.

An additional scenario for how TSD may evolve from GSD might also be considered. Although non-Fisherian sex ratio evolution has long been known to occur for sex chromosomes and extrachromosomal inheritance (Hamilton, 1967), models of non-Mendelian inheritance patterns leading to skewed primary sex ratios have only recently been considered in the context of reptiles with ESD (Freedberg and Wade, 2001). The possibility that ESD may have arisen simply as a sex-ratio distorter has not been investigated in this context, although it is likely to have played a major role in the evolution of a form of ESD in some invertebrates (Rigaud et al., 1997). With these additional possibilities in mind, I evaluated the possibility of ESD arising and being maintained as a relatively neutral change in sex determining system, and also the possibility of ESD arising as a sex ratio distorter in populations with GSD. For one scenario, I also demonstrated the results by algebraically deriving an analytical model in addition to the simulations. Although much of the theoretical groundwork for sex ratio evolution has been established by Karlin and Lessard (1986), evaluating equilibrium ratios and effects of temporal variation on the evolution of

sex ratios in ESD systems are exceedingly complex and may be assessed more expediently through simulation.

## Methods

The simulation model is based on a previous model (Morjan, 2002) following basic life history parameters of the painted turtle (*Chrysemys picta*), a common reptile with TSD. The yearly life cycle for the simulation model is shown in Figure 1. Nest temperatures were modeled as a function of two variables: choice of microhabitat by females, and yearly climatic condition. Each year, climatic condition was calculated as a random variable from a normal distribution (SD = ± 1.1°C) taken from long-term climatic data at the nesting site of a thriving population of painted turtles (Janzen, 1994). Nest temperature for each clutch was adjusted from the climatic condition for that year by the choice of microhabitat by the female [by a maximum of ± 2 °C, taken from field data (Janzen and Morjan, 2001; Morjan and Janzen, unpubl. data)].

Three scenarios of nest-site inheritance were investigated. In simulations where microhabitat choice by females was heritable, the sum of four diallelic loci offset each female's mean choice of microhabitat from the original population mean (centered around zero), with a variance determined by one additional diallelic locus. In simulations where choice of microhabitat was not heritable, offspring were assigned random values at the loci so that each cohort mean was always centered around zero. In simulations involving natal philopatry to nest sites, each female oviposited in microhabitats with a mean of her own natal microhabitat value, with a maternally inherited variance similar to the heritable model.

Populations originally exhibited genotypic sex determination with either female (ZZ/ZW) or male (XX/XY) heterogamety. ESD was introduced as a mutation on one of the following chromosomes: extrachromosomal (mitochondrial), on the X or Z chromosome, on the Y or W chromosome, or autosomal. The degree of dominance of ESD over the sex chromosomes was varied such that ESD could be recessive to W or Y, causing only homogametic sexes to reverse sex in certain thermal conditions (Figure 2A; following Bull, 1981). Alternatively, ESD could be dominant to the W or Y chromosome, causing either genotypic sex to reverse sex (Figure 2B). When ESD is dominant, YY or WW combinations were assumed to be lethal. This case assumes that the Y or W chromosome has already accumulated deleterious recessive alleles, expected to be an early consequence of reduced recombination between the sex chromosomes (Charlesworth, 1991).

Mutants with ESD inherited a threshold temperature that was compared to the nest temperature. Two scenarios of threshold temperatures were investigated to evaluate whether genetic variation in this trait was required for ESD to evolve: threshold temperatures were either fixed (at T values producing on average <50%, 50%, or >50% females), or determined by the sum of autosomal modifier genes of small effect (four diallelic loci). Individuals with ESD in a nest with a higher temperature than the threshold temperature became females, whereas those in nests with lower temperatures became males.

Offspring survivorship during the first year was ~0.05 and after the first year remained at ~0.85 per year for both sexes in the initial simulations. Some later simulations used an adult survivorship rate of 0.95 for reasons described below. Two scenarios were investigated: equal offspring mortality for both sexes, or employing a "differential fitness"

function enhancing fitness as incubation temperature decreased for males and increased symmetrically for females.

Ten simulations were conducted for each scenario. Due to the large number of permutations required to test every possible scenario (576; not all permutations were possible, such as an ESD mutation on a Y chromosome being recessive to a Y chromosome), a high mutation rate was initially used such that a transmitted allele had a 0.01 probability of mutating into its alternative state (ESD or loss of ESD). This way, conditions where ESD is not expected to evolve could be eliminated rapidly to conserve computing time. Selected scenarios where ESD successfully invaded GSD were run again at lower mutation rates ($10^{-3}$, $10^{-4}$, $10^{-5}$), and one scenario was evaluated further by developing an analytic model.

## Results

Results for the first round of simulations (mutation rate = 0.01) are summarized in Tables 1-4. Several scenarios existed in the first round where ESD was maintained at high frequencies with GSD that did not include the differential fitness function or the two other conditions necessary in Bull's (1981) model. In some cases the W or Y sex chromosome was eliminated entirely.

Generally, the most successful scenarios (those eliminating W or Y chromosomes) were where ESD (1) was extrachromosomally inherited, (2) biased sex ratios to produce a majority of the sex that was originally heterogametic, (3) was dominant to the Y or W chromosome, and (4) two isolated cases involving natal philopatry. The least successful

scenario was generally if ESD resided on the Y or W chromosome (N = 36 batches, frequency W or Y: 0.24 ± 0.01, frequency of ESD: 0.02 ± 0.01).

The following scenarios (highlighted in bold in Tables 1 – 4) were examined in more detail.

*1. Extrachromosomal inheritance, female heterogamety, ESD recessive to W:*

When the mutation rate was decreased to $10^{-4}$, ESD successfully invaded in all 5 of these simulations within 7000 – 25000 generations, and reached fixation at 7000 years in the single simulation conducted with the mutation rate reduced to $10^{-5}$. An analytical model provided in Appendix 1 indicates that ESD will always invade and be maintained as an extrachromosomal mutation (assuming perfect transmission) if the environment produces ≥ 50% female offspring. If exactly 50% of the environment produces female offspring, ESD will reach fixation in a ZW system.

*2. Simulations where ESD was maintained at an equilibrium with GSD.*

Specifically, I focused on the scenario where ESD occurs as an autosomal mutation, 50% of the environment is expected to produce females, and ESD is recessive to the existing genotypic sex determining system (Table 3, row 2). In the initial results, ESD was expected to reduce the frequency of W by half. However, when mutation rates (gain and loss of ESD) were lowered to $10^{-4}$, the fitness costs of ESD vs. GSD became apparent (Figure 3A). As sex ratio of the adult population began to increase in variance from 0.5, the frequency of ESD declined. ESD frequencies tended to spike rapidly and just as rapidly die off.

Previous simulation and analytical models suggest that the fitness disadvantage of ESD is reduced when the expected survivorship of individuals is increased (Bull and Bulmer, 1989; M. Girondot, unpubl. data). When adult survivorship was accordingly increased to 0.95 for both sexes, ESD could again be maintained for longer periods with GSD at this smaller mutation rate (Figure 3B). In 4 out of 10 simulations, GSD was even replaced entirely by TSD (however, one of these 4 populations subsequently went extinct). TSD increased in frequency whether genetic variation for threshold temperature was present (Figure 3C) or not (Figure 3B).

*3. Natal philopatry (ESD recessive to W).*

When mutation rates were reduced to $10^{-4}$, ESD successfully eliminated W chromosomes in all of 10 simulations involving female heterogamety and natal philopatry to nest sites. Time to extinction of the W chromosome was quite variable (Figure 4; mean year ± SD to extinction: 236,000 ± 142,400). However, allele frequencies of ESD were maintained close to 0.5, indicating that a large proportion (~25%) of the population consisted of ZZ males without ESD (mean frequency of ESD at the end of 10 simulations: 0.53 ± 0.05 SD). These results suggest that ESD in this system cannot completely replace GSD, but simply replaced W chromosomes. Results were similar if ESD occurred on the Z chromosome rather than an autosome (W went extinct in 5 out of 6 simulations, mean year ± SD to W extinction: 202,000 ± 79,000, frequency ESD = 0.55 ± 0.03).

*4. Dominance of ESD over Y/W.*

ESD clearly invaded GSD in the initial simulations: as ESD increased, Y or W chromosomes were eliminated through the production of homozygous lethals (Table 4). However, if the mutation rate was lowered to $10^{-4}$ (with survivorship left at 0.85) it became apparent that the location of the ESD mutation was important for its success in invasion. ESD still rapidly invaded in 10 out of 10 simulations if the mutation occurred on the X or Z chromosome [Figure 5A; mean years (± SD) to extinction for the W or Y chromosome: 11,100 ± 4,600]. In contrast to the initial results (which were run for very few generations), ESD reached fixation in all of these scenarios with little displacement from loss-of-ESD mutations (Figure 5A). On the other hand, GSD remained the predominant sex determining mechanism if ESD occurred on an autosome instead (Figure 5B).

## Discussion

If ESD is derived, it does not necessarily imply that ESD is adaptive to particular life history traits. Rather, a more complete view might focus on the potential disadvantages of ESD compared to GSD (Girondot and Pieau, 1999). One possible disadvantage is that ESD may produce intersexes (Bull, 1983). However, intersexes have been rarely observed and found to be generally transient in turtles with TSD (Crews et al., 1994; Girondot et al., 1997). The more plausible disadvantage of ESD is the tendency for temporal variation in environmental conditions to skew sex ratios, even if conditions produce a 1:1 sex ratio on average (Bull, 1980). For non-overlapping generations, major sex factors are expected to increase in populations experiencing temporal environmental variation (Bull, 1983). However, certain life history traits such as longevity and a large number of overlapping

generations may in fact allow ESD to persist or evolve as a relatively neutral mechanism (Bull and Bulmer, 1989; Giondot and Pieau, 1999; M. Girondot, unpubl. data). Indeed, many turtles exhibit survivorship rates as high as 0.9 (Iverson, 1991; Wilbur 1975; Mitchell 1988; Zweifel 1989; Iverson and Smith 1993). In the neutral model (scenario 2), changes in survivorship rates from 0.85 to 0.95 determined whether ESD could successfully evolve and be maintained in a GSD population experiencing realistic spatial and temporal environmental variation. However, this model required that the temporal environmental variation fluctuated around a constant mean; changes in this mean over time would certainly have increased the disadvantages of ESD. Although primary sex ratios are predicted to fluctuate around constant means in freshwater (Janzen, 1994) and marine turtles (M. Girondot, pers. comm.), these long-term studies are in reality short-term regarding the potential neutrality of ESD. My simulations demonstrate that ESD could possibly be maintained in a population of individuals experiencing long life spans, but a long period of climatic stasis is also likely to be necessary. They also predict that if ESD was a relatively neutral mechanism and recessive to the effects of Y or W, heteromorphic sex chromosomes may also be maintained in the population for a long time. This result is inconsistent with the general dichotomy between reptilian taxa exhibiting heteromorphic sex chromosomes and those exhibiting TSD (Bull, 1980).

Sex ratios from eggs incubated under constant conditions exhibit a strong clutch-of-origin component in turtles and crocodilians with TSD (Bull et al., 1982; Janzen, 1992; Rhen and Lang, 1998), suggesting some genetic contribution to sex in addition to thermal conditions. Girondot and Pieau (1999) argue that this genetic contribution to sex determination in reptiles with TSD provides evidence against the Charnov-Bull hypothesis.

If the differential fitness condition held, individuals with genetic tendencies to become a particular sex are likely to become the sex of lower fitness for their environmental conditions. Therefore, any genetic tendency to become a particular sex would be selected against. They conclude that the prevalence of genetic contributions to sex determination suggests that ESD is relatively neutral or has simply not been selected against, rather than adaptive (Girondot and Pieau, 1999). In the initial simulations, the evolution of ESD did not require widespread pre-existing genetic variation (Bull, 1981) or a lack in genetic variation (Woodward and Murray, 1993; see Girondot and Pieau 1996) in environmental sensitivity for sex determination. A model based on life-history data of crocodilians concluded that ESD would produce female-biased sex ratios and be maintained, but populations exhibiting GSD would go extinct (Woodward and Murray, 1993). Two major assumptions of Woodward and Murray's (1993) model included natal philopatry to nesting sites, and female-limited population dynamics such that the population would go extinct if the number of females fell below a threshold value. Reanalysis of their model by simulation demonstrated that when genetic polymorphism for ESD was introduced, male-biasing alleles would invade the population and cause extinction under ESD (Girondot and Pieau, 1996). Such a problem is unlikely in my simulations because no differences in outcomes for the initial simulations occurred when including genetic variation in ESD (see SD for combined results in Tables).

Several models concerning the adaptive significance or evolutionary dynamics of ESD rely on natal philopatry (Reinhold, 1998; Woodward and Murray, 1993; Freedberg and Wade, 2001). Reinhold (1998) demonstrated that ESD may be selected for through natal philopatry because of uneven inheritance patterns between sons and daughters. In this case,

a daughter inherits the nest site from her mother in addition to genetic contributions from both parents. This model includes a differential fitness function because fitness costs are greater for females produced at low-quality nest sites than for males, since offspring of those females will also develop in the low-quality sites. ESD would then be selected by causing high-quality sites to produce a majority of female offspring, and low-quality sites to produce males. Although this model provides a novel measure of how fitness by environmental condition may vary between males and females, it has not been supported by a recent empirical study (Valenzuela and Janzen, 2001).

One of the major problems with models concerning sex-ratio skewing through philopatry and ESD is that they do not tend to be evolutionarily stable. Girondot and Pieau (1996) further demonstrated that genetic variation in female nesting behavior disrupted the maintenance of ESD in the model proposed by Woodward and Murray (1993). Similar to the scenario where genetic polymorphism for ESD was introduced, if females exhibited genetic variation in their nesting preferences, the population went extinct due to selection for females to nest in male-producing areas. This instability is also apparent in a model by Freedberg and Wade (2001) demonstrating the tendency for natal philopatry to cause female-biased sex ratios in populations exhibiting ESD. This tendency is expected to occur because sites producing females are the sites where most individuals will return to nest in subsequent generations, causing a runaway process favoring females. Alleles causing male-biased offspring sex ratios are then expected to increase in frequency to counteract the effects of natal philopatry. For the model to be evolutionarily stable, Freedberg and Wade (2001) introduce periodic environmental disruptions causing females to nest in new sites. However, if no such disruption occurs, genetic variation favoring males is likely to be

depleted and philopatry may eventually cause extinction through female-biased sex ratios. In my initial simulations, ESD combined with natal philopatry initially caused a major decrease in W, the major female-determining gene (Tables 2 and 3). However, this condition could not persist without GSD because of selection for ZZ males that did not carry ESD (Figure 4).

One scenario where ESD completely eliminated GSD involved unequal maternal and paternal contributions to offspring (Appendix 1). Although sex ratios in vertebrates are unlikely to be affected by extrachromosomally inherited elements (Hurst, 1993), this scenario demonstrated that ESD could rapidly and successfully invade a GSD population without any adaptive significance. In other models, extrachromosomal sex ratio distorters produce females rather than having thermally-dependent expression (Bull, 1983; Taylor, 1990). Equivalent dynamics to my TSD model are produced through varying the transmission efficiency or penetrance of the mutation. Previous models show that extrachromosomal sex ratio distorters will always invade a population if the frequency of females produced by carriers of the mutation is greater than the frequency of females produced by non-carriers (Bull, 1983; Taylor, 1990). The invasion occurs because extrachromosomal material (e.g., mitochondria or cytoplasmic elements) are inherited through the mother. Extrachromosomal elements biasing sex ratios toward females will increase in frequency compared to those that are transmitted equally between males and females, because those ending up in males cannot be transmitted. However, my models demonstrate that the mutation can increase even if the TSD mutation alone causes a 1:1 primary sex ratio. This will only occur in a ZW sex determining system rather than a XY system because carrier ZW females will produce 50% females through transmitting the W

chromosome, but also some WW females from the carrier mutation. As long as the W chromosome exists, the extrachromosomal mutation will increase in frequency at expense of W. The two sex determining systems in combination initially bias sex ratios toward females, and after the W chromosome is eliminated, sex ratios again are maintained at 1:1. This scenario demonstrates that ESD could rapidly overtake a GSD system without any adaptive significance.

A similar phenomenon has been demonstrated in invertebrates, particularly in populations infected with a strain of bacteria (*Wolbachia*) that distorts sex ratios. For example, in isopods (*Armadillidium vulgare*) and the woodlouse (*Porcellionides pruinosus*), highly female-biased sex ratios result when offspring develop at ~20°C, but highly male-biased sex ratios are produced when they are reared at ~30°C, suggesting that they exhibit ESD (Rigaud et al., 1991; 1997). On further investigation, some *A. vulgare* populations were shown to consist completely of ZZ genotypic males, many of which had been infected with a feminizing bacteria belonging to the genus *Wolbachia* (Rigaud, 1991). Similar to my analytical model, the W chromosome is thought to have been eliminated from these populations due to the feminizing effect of *Wolbachia* (Rigaud et al., 1991; 1997). The bacteria are either killed or lose their sex-biasing effectiveness at high temperatures; hence the reason that males are produced at high temperatures. Therefore, these invertebrates do not exhibit ESD in the strict sense, rather ESD is a product of incomplete sex reversal under cytoplasmic sex determination (Rigaud et al., 1997). Furthermore, Rigaud et al. (1997) suggest that there is no adaptive significance to this system based on observations of the life history of infected individuals.

If ESD has (had) an adaptive advantage over GSD, it may not involve matching offspring fitness to environmental patches, but instead with reducing fitness of individuals with sex chromosomes by interfering with the original sex-determining system. This possibility is demonstrated by the alternative (but not previously examined) scenario proposed by Bull (1981), in which ESD has the potential to cause both genotypic sexes to differentiate into the opposite sex (Figure 2B). Bull (1980) also considered the apparent dichotomy among reptilian taxa exhibiting ESD and those exhibiting heteromorphic sex chromosomes, suggesting that the two sex determining mechanisms are incompatible. He noted that ESD is unlikely to evolve as a mechanism causing XY females because of the fitness cost associated with producing YY offspring. However, Bull (1980) did not consider the possibility that ESD would increase in that scenario if ESD was X linked (Figure 5A).

A similar scenario involving polymorphic sex chromosomes exists in several fishes, including platyfish (Orzack, 1980) and many African cichlids (Lande et al., 2001). Although this system is called W,X,Y in the literature, here it will be called R,X,Y to eliminate confusion with the ZW/ZZ system. In this polymorphic system, R is an X-linked mutation causing XY males to reverse sex. The reason R is frequently X-linked was recently demonstrated by Lande et al. (2001). Since Y tends to accumulate deleterious recessive mutations (Charlesworth, 1991), YY is frequently lethal or at least causes a reduction in fitness. If R arises as a mutation that is not X-linked, it will frequently be associated with YY offspring from R-XY females and XY males, an evolutionary dead-end. R therefore increases in frequency only slightly if not X-linked. However, if R is tightly X-linked, R will not be associated with the YY genotype, and increases in frequency as Y is lost. Since R completely reverses sex in cichlids (Lande et al., 2001), R is selected against

as it increases because it causes increasingly female-biased sex ratios. Therefore, maintaining R requires an introduction of an autosomal repressor and sexual selection for males carrying this repressor (Lande, 2001).

My simulations demonstrated a similar result using a dominant, Z-linked form of ESD, but without the associated cost of sex-ratio selection as ESD increased. ESD increased in frequency when Z-linked, but did not increase if associated with an autosome (Figures 4 and 5). W was eliminated similar to a delayed form (by one generation) of Z-drive when ESD was sex linked, by producing WW lethal genotypes. However, in my simulations ESD experiences only weak sex-ratio selection as it increases in frequency, because ESD causes a nearly 1:1 sex ratio when it is common. Although a second major difference between my model and that of Lande et al. (2001) was that ESD caused both genotypic sexes to reverse sex, the dynamics of increasing ESD through a reduction in fitness of WW individuals are similar.

Realistically, it is unclear whether ESD could arise as a mutation of major effect, as major sex-reversing mutations are not commonly known to be environmentally-sensitive (Bull, 1981). The biological significance of thermal conditions overriding genotype in amphibians has been questioned (Hayes, 1998), although thermal conditions likely to be experienced in nature were recently found to reverse sexes in a newt (Wallace and Wallace, 2000). The production of XY females in that species suggests the possibility of ESD as a dominant sex reversal system may not be too far-fetched. Secondly, without the rapid evolution of autosomal restorers, a dominant ESD mutation may increase so rapidly that genetic polymorphism in sex determination would rarely be detected. For this to happen, the ESD mutation would have to cause no loss in fertility for sex-reversed individuals

(beyond producing homozygous lethals), and be X- (or Z-) linked. Genes located on W or Y chromosomes act as independent and recent solutions for controlling a conserved sex determining mechanism in vertebrates (Nanda et al., 1999); mutations at any other step of the conserved genetic pathway may interfere with or even eliminate the original major sex-determining factor (reviewed in Graves and Shetty, 2001). In fact, ZW sex determination in vertebrates may act to regulate dosage-dependent transcription of a conserved gene in sex determination (DMRT1) on the Z chromosome (Graves and Shetty, 2001), suggesting that genes governing sex determination ultimately reside on Z rather than on W or autosomes in that system. An environmentally-sensitive mutation in a similar sex determining system could cause the dynamics demonstrated in this model.

One caveat in these simulation models was that ESD was introduced as either a gain or loss of function on a specific chromosome. Therefore, in some simulations, the Y or W chromosome was lost but a form of GSD still persisted through homogametic individuals lacking the ESD mutation (Figure 4). Possibly, another mutation of major effect on sex determination could arise before the original Y or W chromosome is lost, preventing ESD from being maintained. Rather than evaluating such possible scenarios (which would be difficult to predict), these models focused on how likely ESD may evolve in an existing GSD system without making assumptions of how a new GSD system may subsequently arise.

These simulations were developed to evaluate alternative mechanisms that may have allowed ESD to evolve and persist in reptiles, without using the typical differential fitness function that has received so much attention despite little empirical support for the past 25 years. In sum, the simulations suggest that ESD may arise and be maintained as a relatively

neutral mechanism in very long-lived organisms. ESD may also arise as a sex ratio distorter, either through changing the proportion of environment (T) producing females or through natal philopatry favoring female production. ESD is likely to coexist with GSD for a long time in either case, ranging from GSD going extinct in the neutral scenario to GSD remaining necessary in the sex-ratio distorter scenario. ESD may also evolve by causing a reduction in fitness for homogametic YY or WW individuals. Of course, these scenarios rest on the assumption that ESD was derived from GSD at some point, rather than persisting as an ancestral condition in vertebrates. Further insight into these issues might be found through phylogenetic analyses and investigating molecular mechanisms of TSD.

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**Table 1.** Frequencies of W/Y and ESD when ESD is extrachromosomally inherited and recessive to W or Y chromosomes. Results for all scenarios of nest-site inheritance and genetically fixed or variable threshold temperatures were combined. Scenarios in bold type were used for further investigation.

| Heterogamety | N | SR bias of ESD | Adult SR (%F) | Freq. W or Y | Freq. ESD |
|---|---|---|---|---|---|
| F | 4* | F | 0.69 ± 0.11 | 0 ± 0 | 0.99 ± 0 |
| **F** | **6** | **1:1** | **0.52 ± 0.05** | **0.02 ± 0.03** | **0.95 ± 0.04** |
| F | 6 | M | 0.51 ± 0.01 | 0.23 ± 0.01 | 0.54 ± 0.06 |
| M | 18 | F/1:1/M | 0.50 ± 0.01 | 0.25 ± 0.01 | 0.03 ± 0.03 |

Heterogamety: F = female (ZW), M = male (XY). N: number of batches of 10 simulations each. SR bias: threshold temperature adjusted to favor females (F), males (M), or both sexes equally (1:1).

* = in two scenarios, all simulations went extinct through excess female-biased SR

**Table 2.** Frequencies of W/Y and ESD when ESD is on a sex chromosome and recessive to W or Y. Results for genetically fixed and variable pivotal temperatures were combined. SD $\leq$ 0.02 unless otherwise indicated.

| Chromosome | N | SR bias | Nest-site inheritance | Adult SR (%F) | Freq. W or Y | Freq. ESD |
|---|---|---|---|---|---|---|
| Z | 6 | F | N/H/P | 0.51 | 0 | 0.32 |
| Z | 4 | 1:1 | N/H | 0.50 | 0.12 | 0.39 ± 0.03 |
| Z | 2 | 1:1 | P | 0.48 | 0.05 | 0.43 |
| Z | 6 | M | N/H/P | 0.50 | 0.22 | 0.38 |
| X | 6 | F | N/H/P | 0.49 | 0.23 | 0.40 |
| X | 4 | 1:1 | N/H | 0.48 | 0.12 | 0.39 |
| X | 2 | 1:1 | P | 0.51 | 0.16 | 0.41 |
| X | 6 | M | N/H/P | 0.50 | 0 | 0.33 |

Chromosome: chromosome where ESD mutation was introduced. N: number of batches of 10 simulations each. SR bias: threshold temperature adjusted to favor females (F), males (M), or both sexes equally (1:1). Nest-site inheritance: N= no inheritance, H= biparental inheritance, P = natal philopatry.

**Table 3.** Frequencies of W/Y and ESD when ESD is on an autosomal allele and is recessive to W or Y. Results for genetically fixed and variable pivotal temperatures were combined. SD < 0.01 for all values. Scenarios in bold type were used for further investigation.

| Heterogamety | N | SR bias | Nest-site inheritance | Adult SR | Freq. W or Y | Freq. ESD |
|---|---|---|---|---|---|---|
| F | 6 | F | N/H/P | 0.51 | 0 | 0.31 |
| **F** | **4** | **1:1** | **N/H** | **0.50** | **0.12** | **0.42** |
| **F** | **2** | **1:1** | **P** | **0.51** | **0.05** | **0.44** |
| F | 6 | M | N/H/P | 0.50 | 0.22 | 0.48 |
| M | 6 | F | N/H/P | 0.50 | 0.23 | 0.48 |
| M | 4 | 1:1 | N/H | 0.50 | 0.11 | 0.42 |
| M | 2 | 1:1 | P | 0.51 | 0.16 | 0.44 |
| M | 6 | M | N/H/P | 0.49 | 0 | 0.32 |

Heterogamety: F = female (ZW), M = male (XY). N: number of batches of 10 simulations each. SR bias: threshold temperature adjusted to favor females (F), males (M), or both sexes equally (1:1). Nest-site inheritance: N= no inheritance, H= biparental inheritance, P = natal philopatry.

**Table 4.** Frequencies of W/Y and ESD when ESD is dominant to the existing genotypic sex determining system. All forms of nest-site inheritance and genetic variation for threshold temperatures were combined for each row. N= 6 batches of 10 simulations each for each row. SD < 0.02 unless indicated otherwise. This table represents the scenarios where female heterogamety was the existing genotypic sex determining system; male heterogamety followed similar patterns (except for two cases of natal philopatry where ESD failed to invade). Scenarios in bold type were used for further investigation.

| Location of ESD | SR bias of ESD* | Adult SR | Freq. W | Freq. ESD |
|---|---|---|---|---|
| Autosome | F | 0.51 | 0 | 0.31 |
| | **1:1** | **0.49** | **0** | **0.54 ± 0.09** |
| | M | 0.46 | 0.25 | 0.06 |
| Z | F | 0.50 | 0 | 0.32 |
| | **1:1** | **0.48 ± 0.03** | **0.01** | **0.55 ± 0.06** |
| | M | 0.43 | 0.24 | 0.15 ± 0.05 |

*SR bias of ESD: threshold temperature adjusted to favor females (F), males (M), or both sexes equally (1:1).

**Figure 1.** Life cycle of individuals with TSD used in the simulation models.

**Figure 2.** Scenarios used in modeling ESD as a sex reversing gene in a GSD population. T is the proportion of the environment causing an individual to become female under ESD. The ZW system is shown, but the same design is also used for the XY system. **A)** ESD is recessive to W, causing homozygotic individuals to reverse sex. **B)** ESD is dominant to both sex chromosomes, causing either genotypic sex to differentiate into the opposite sex.

**Figure 3.** Mean frequencies of W chromosome and ESD allele, and mean adult sex ratio (frequency of females) in 10 simulations when ESD is introduced as an autosomal mutation ($10^{-4}$) and recessive to the W chromosome. SE are indicated by dotted lines. T = 0.50 (environment expected on average to produce a 1:1 sex ratio under ESD). **A)** Survivorship for males and females is 0.85. **B)** Survivorship is increased to 0.95. **C)** Same as B (S = 0.95) but including genetic variation for threshold temperatures.

**Figure 4.** Mean frequencies of W chromosome and ESD allele, and mean adult sex ratio (frequency of females) in 10 simulations with female heterogamety and when females are philopatric to nest temperatures. SE are indicated by dotted lines. ESD is introduced as an autosomal mutation ($10^{-4}$), T = 0.50, and survivorship = 0.95.

**Figure 5.** Mean frequencies of W chromosome and ESD allele, and mean adult sex ratio (frequency of females) in 10 simulations each where ESD is dominant to the W

chromosome in a ZW GSD system. SE are indicated by dotted lines. Survivorship = 0.95. **A)** ESD is located on the Z chromosome. **B)** ESD is located on an autosome.

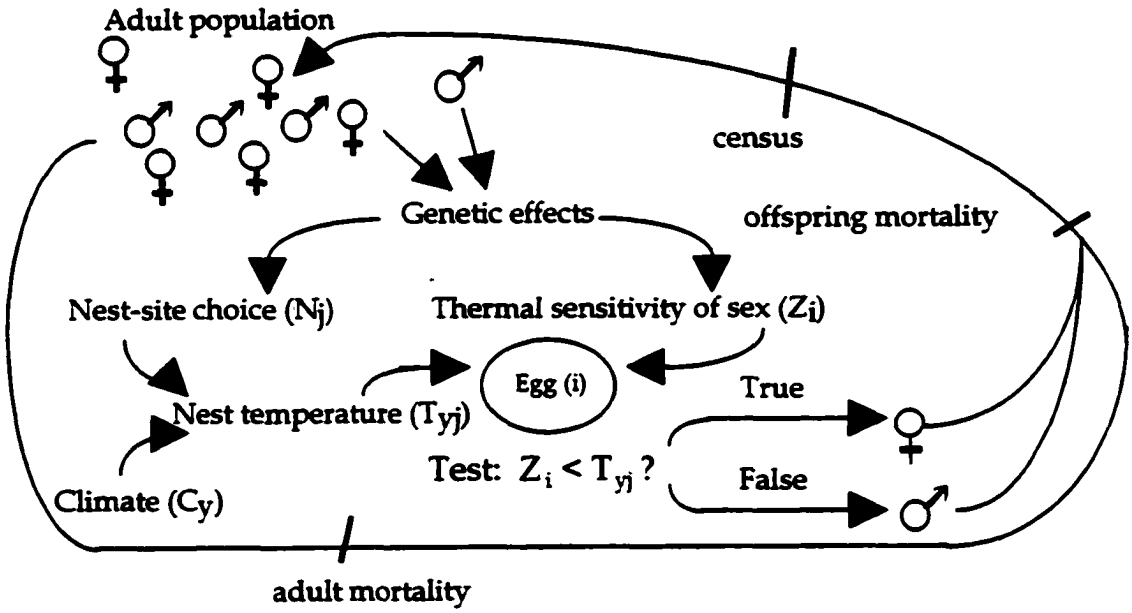

Adult population
census
Genetic effects
offspring mortality
Nest-site choice (Nj)
Thermal sensitivity of sex (Zi)
Egg (i)
Nest temperature (Tyj)
True
Test: Zi < Tyj ?
False
Climate (Cy)
adult mortality

**Figure 1.**

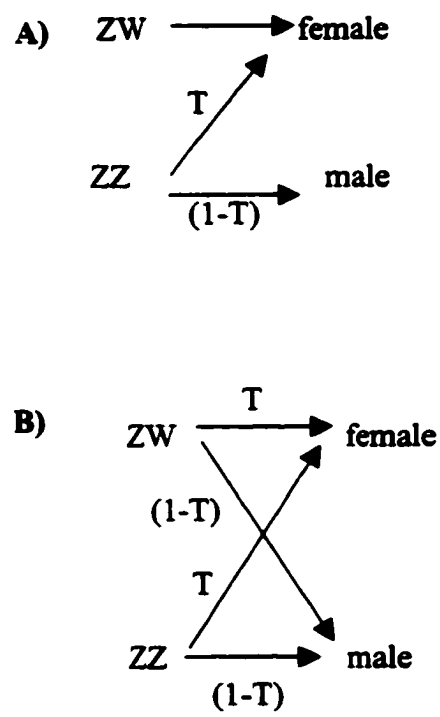
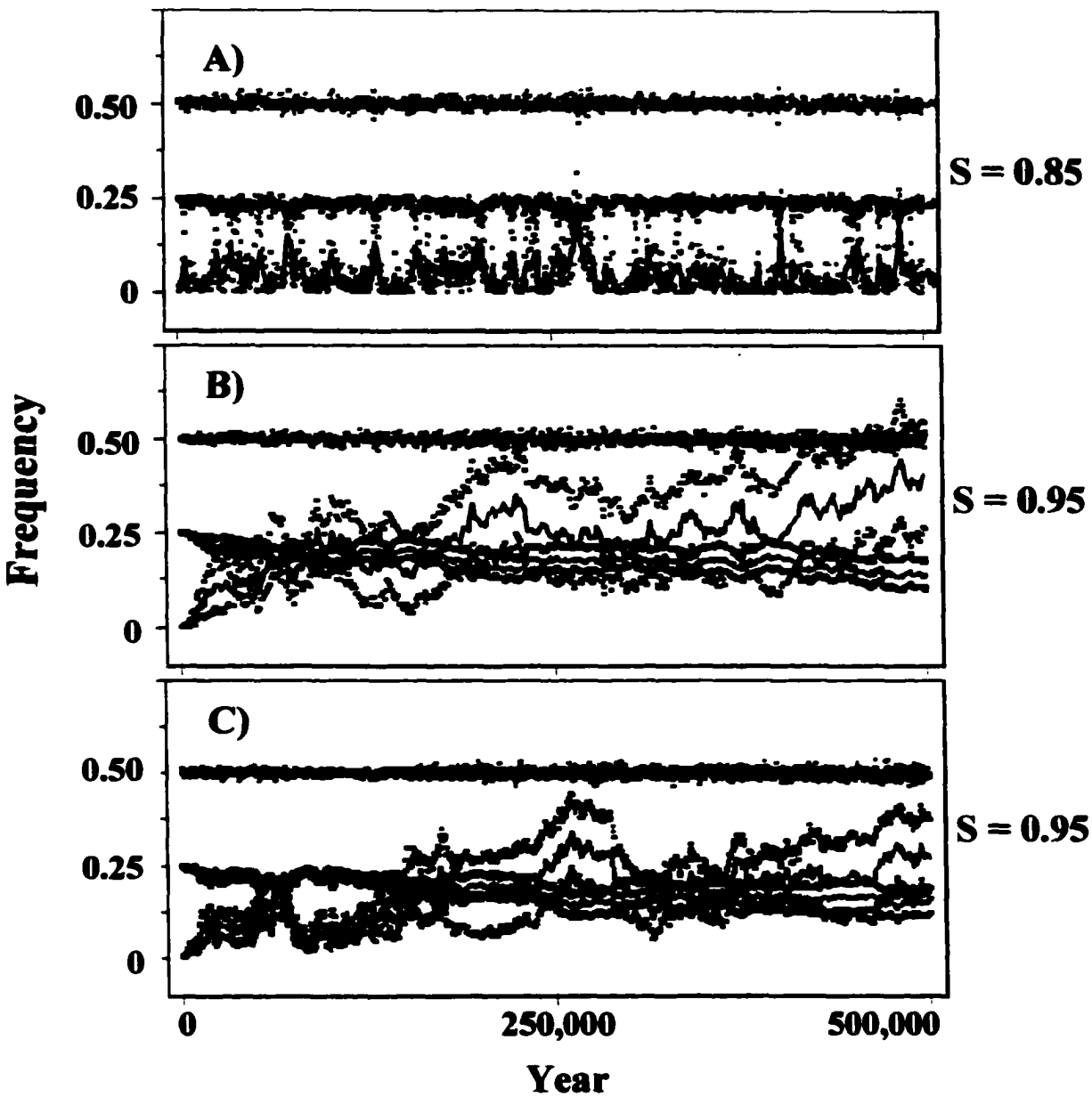



**Figure 2.**


A)
B)
C)
0.50
0.25
0
S = 0.85
S = 0.95
S = 0.95
Frequency
0
250,000
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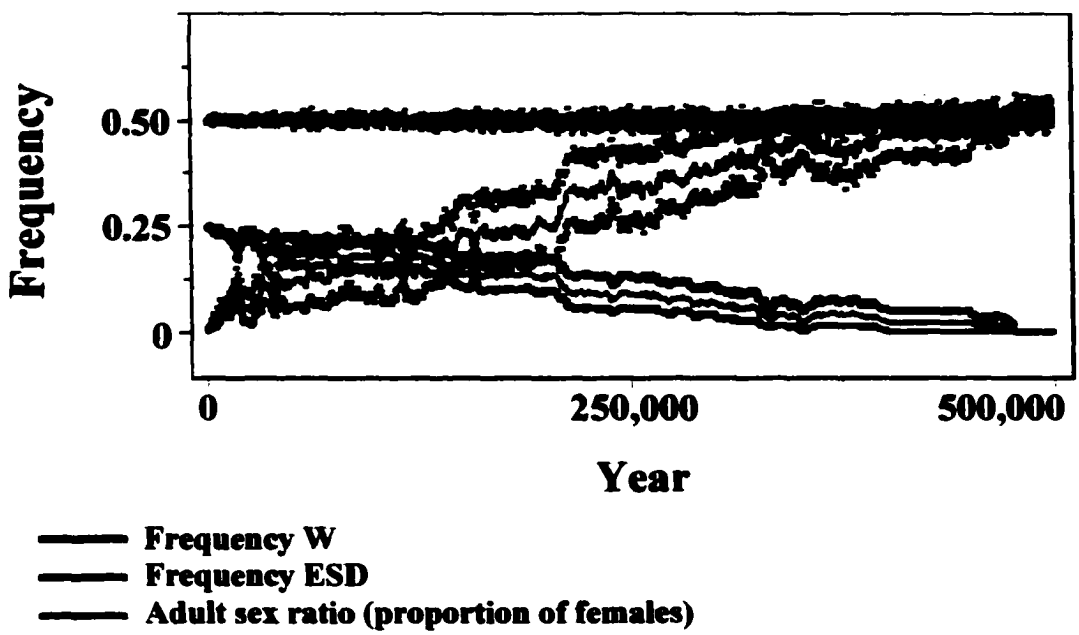
Year
Frequency W
Frequency ESD
Adult sex ratio (proportion of females)

**Figure 3.**


Frequency
0.50
0.25
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250,000
500,000
Year
Frequency W
Frequency ESD
Adult sex ratio (proportion of females)

**Figure 4.**

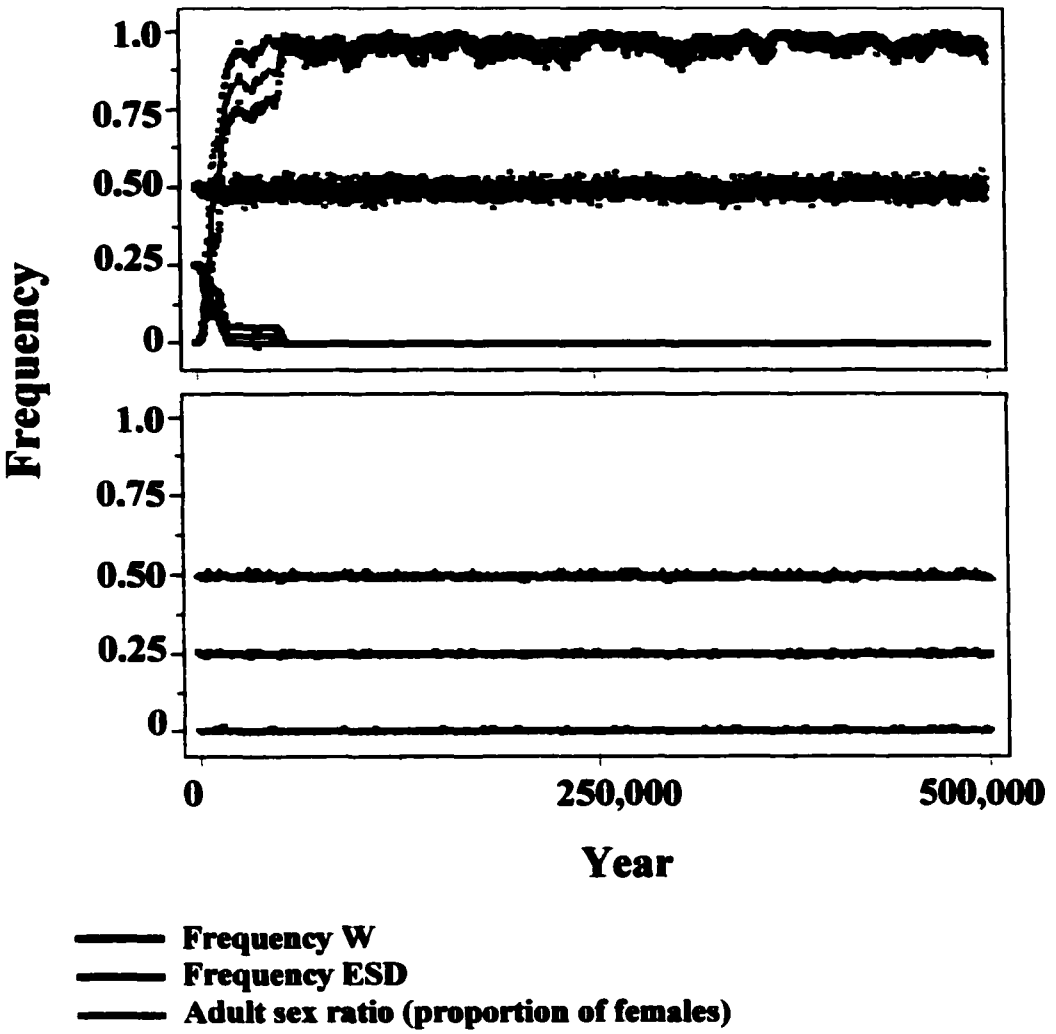
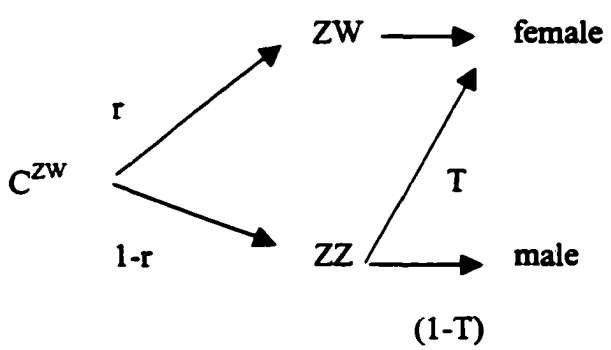

1.0
0.75
0.50
0.25
0
Frequency
1.0
0.75
0.50
0.25
0
0
250,000
500,000
Year
Frequency W
Frequency ESD
Adult sex ratio (proportion of females)

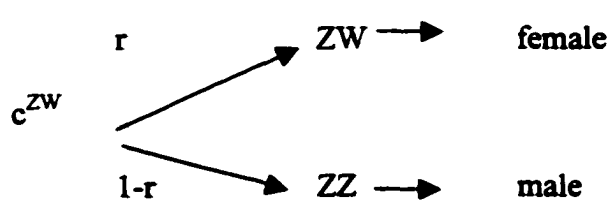
**Figure 5.**

**Appendix 1.**

**ESD under cytoplasmic inheritance.**

Suppose a population exhibits GSD such that females are heterogametic. A proportion of females exhibits the cytoplasmic sex ratio distorter at frequency $p$. The remaining females exhibit GSD at the frequency $q = 1 - p$. The sex ratio of offspring (proportion of females) produced by carrier females (C) is $r_c$ whereas the sex ratio of offspring produced by GSD females (c) is $r$. The ESD mutation causes a fraction (T) of the homozygous offspring to become females rather than males.




The fraction of female offspring in the population (F) is

$$F = rq + r_c p .$$

The frequency of carrier females in the next generation ( $p'$ ) is

$$p' = \frac{r_c p}{F} .$$

The frequency $p$ will increase when $p' > p$, such that $\frac{p'}{p} > 1$:

$$\frac{r_c p}{Fp} > 1$$

$$\begin{aligned} r_c &> rq + r_c p \\ r_c &> r(1-p) + r_c p \\ r_c - r_c p &> r(1-p) \\ r_c(1-p) &> r(1-p) \\ r_c &> r. \end{aligned}$$

On the other hand, the fraction of female offspring in the population can also be defined as the weighted average of the sex ratios from females carrying W chromosomes and those females not carrying a W chromosome.

$$F = wr_G + (1-w)T ,$$

where w is the fraction of females carrying the W sex chromosome, and $r_G$ is the sex ratio produced by those females. Note that $r_G \neq r$ because $w$ may include carrier females.

The frequency of W females in the next generation ( $w'$ ) is

$$w' = \frac{wr}{F} .$$

For $w$ to decrease, $\frac{w'}{w} < 1$ so

$$\frac{wr}{Fw} < 1$$
$$r < F$$

since $F = rq + r_c p$,

$$r < rq + r_c p$$
$$r < r(1-p) + r_c p$$
$$\cdot$$
$$\cdot$$
$$\cdot$$
$$r < r_c$$

Therefore, when $p$ increases, $w$ also decreases.

Now, $r_c$ is the weighted average of the sex ratios produced by $C^{ZZ}$ females and $C^{ZW}$ females.

$$r_c = \frac{(1-w)T}{p} + [1 - \frac{(1-w)}{p}][r + (1-r)T].$$

For $r_c > r$ then

$$\frac{T(1-w)}{p}+[1-\frac{(1-w)}{p}][r+T(1-r)]>r$$

$$\frac{T(1-w)}{p}+r+T(1-r)-\frac{r(1-w)}{p}-\frac{T(1-w)(1-r)}{p}>r$$

$$\frac{T(1-w)-r(1-w)-T(1-r)(1-w)}{p}>r-r-T(1-r)$$

$$T(1-w)-r(1-w)-T(1-r)(1-w)>-pT(1-r)$$

$$(1-w)[T-r-(1-r)T]>-pT(1-r)$$

$$(1-w)(T-r-T+Tr)>-pT(1-r)$$

$$(1-w)(Tr-r)>-pT(1-r)$$

$$r(1-w)(T-1)>-p(1-r)T$$

$$-1(T-1)r(1-w)<p(1-r)T$$

$$r(1-w)(1-T)<p(1-r)T$$

$$\frac{r(1-w)(1-T)}{T(1-r)}<p.$$

When $r = 0.5$, this represents a three-dimensional surface:

$$\frac{(1-w)(1-T)}{T}<p$$

Anything below the plane $p$=1 and above the surface of the joint distribution of $T$ and $w$ are feasible values of $T$ and $w$ for ESD to invade. If T = 0.5, $r_c < r$ when

$$1-w<p\,.$$

In this case, $w$ will still approach 0, and $p$ approaches 1, because 1-$w$ (females lacking $w$, or ZZ females) is contained in $p$ (females with ESD mutation) and therefore is always less than $p$.

# CHAPTER 7.

# GENERAL CONCLUSIONS

The evolution and persistence of temperature-dependent sex determination (TSD) in reptiles remains an enigmatic but fascinating avenue for scientific inquiry. Fisher (1930) proposed that primary sex ratios are expected to evolve to 1:1 due to frequency-dependent selection for genes producing the rarer sex. Although a nearly 1:1 sex ratio is already produced through Mendelian segregation of chromosomes in most vertebrates (Williams, 1979; Toro and Charlesworth, 1982), reptiles with TSD lack this constraint and produce biased primary sex ratios depending on thermal incubation conditions (Bull, 1980; Paukstis and Janzen, 1990). Fisher's principle has been demonstrated in some fish (Conover and Van Voorhees, 1990; Basolo, 1994) and experimental strains of *Drosophila* (Blows et al., 2001; Carvalho et al., 1998). There is little evidence to the contrary of why Fisherian sex ratio selection should not also prevail in reptiles with TSD. Skewed population sex ratios are not a rule among these taxa (e.g., Janzen, 1994a; Lance et al., 2000; but see Freedberg and Wade, 2001), and tenable adaptive explanations that could counteract Fisherian sex ratio are lacking (Burke, 1993; Shine, 1999). Selection on heritable variation in two main traits are thought to allow sex ratios to evolve in TSD: maternal nesting behavior affecting nest temperatures, and offspring sex in response to temperature (Bulmer and Bull, 1982). The purpose of the first four studies was to evaluate the selective potential for both of these traits using the painted turtle (*Chrysemys picta*), a common and widespread reptile with TSD. The fifth and final study evaluates the adaptive significance of TSD in a genotypic sex determining system.

The first study was designed to evaluate a key nesting behavior, "ground-nuzzling," in female turtles as a potential thermosensory cue. Although this behavior is widely exhibited among many chelonian taxa (Chapter 2, Table 1), its role in nesting had never previously been investigated. We found that ground-nuzzling was not associated with soil surface temperatures for nesting females. Contrary to observations on sea turtles (Stoneburner and Richardson, 1981), female painted turtles did not nest in sites exhibiting warmer temperatures than other sites encountered during the nesting foray. Furthermore, a thorough review of the literature shows that females in some taxa exhibit ground-nuzzling before emerging from the water, which further suggests against its role as a thermosensory cue. Therefore females either do not use this behavior to detect temperatures, or use it to nest in sites exhibiting intermediate temperatures rather than extremes. Either outcome suggests that this maternal behavior is not used for influencing offspring sex ratios. Further studies evaluating this behavior as an olfactory cue may be profitable.

The second study evaluated geographic variation in offspring sex as a function of temperature between two populations of *Chrysemys picta bellii* inhabiting diverse climatic conditions (Illinois and New Mexico). If pivotal temperatures (the temperature producing a 1:1 sex ratio for each population) are concordant with climatic condition, variation in thermal response of offspring sex may be an important component in the maintenance of sex ratios in this species. Pivotal temperatures indeed were statistically significantly higher in the New Mexico population than in Illinois; however the magnitude of this difference was small. Maternally-derived yolk steroid hormones are correlated with sex ratios in some turtles (Janzen, 1998; Bowden, 2001). However, eggs from New Mexico exhibited higher yolk estradiol concentrations and estradiol:testosterone (E:T) ratios than those from Illinois,

opposite to the direction expected from known effects of estradiol on sex determination in *C. picta*. These results suggest that variation in pivotal temperatures may in part contribute to the maintenance of sex ratios; however this variation is not readily explained by differences in yolk steroid concentrations.

The third study investigated geographic variation in nesting behavior and effects on nest temperatures between two populations of *Chrysemys picta bellii* inhabiting diverse climatic conditions (Illinois and New Mexico). Although nesting behavior by females is thought to be an important mechanism for the maintenance of sex ratios in reptiles with TSD, no previous study has evaluated this conjecture empirically. Vegetation cover over nests has received considerable attention as a microhabitat variable associated with nest temperatures in studies on nest-site selection in turtles (Vogt and Bull, 1984; Janzen, 1994b; Roosenburg, 1996; Wilson, 1998; Weisrock and Janzen, 1999; Janzen and Morjan, 2001). Therefore, if females use nest-site selection to maintain similar nest thermal environments across populations, I expected females in New Mexico to nest under relatively greater vegetation cover than females in Illinois. However, nesting patterns in New Mexico were most highly correlated with maximizing soil moisture around nests, which also kept nests relatively cool compared to their surroundings. Mean nest temperatures in New Mexico were similar to those in Illinois. These results suggest that nest-site selection by females is likely an important component in maintaining sex ratios across populations. However, such patterns may not be a result of sex-ratio selection, but may primarily reflect nest-site selection for microhabitats (e.g., hydric conditions) affecting other offspring traits.

The fourth study used simulation models based on a natural population of painted turtles to evaluate the relative evolutionary potential for female nest-site selection and

pivotal temperatures in maintaining sex ratios. Nesting behavior by females has been widely thought to have a more important role than thermal sensitivity of offspring sex in the maintenance and evolution of sex ratios in reptiles with TSD (Bull et al., 1982a; 1982b; Bulmer and Bull, 1982; Vogt and Bull, 1982; Ewert et al., 1994; but see Rhen and Lang, 1998). In contrast, the simulation models predict that females had little control over the sex ratios of their offspring, based on the relatively large influence of climate compared to microhabitat selection by females on nest temperatures. Thermal sensitivity of offspring sex evolved more rapidly in the models than did nest-site selection by females. These results predict that females have lower potential to adaptively adjust sex ratios through nest-site choice in this system than has previously been thought. Further studies could focus on how temperature affects hatching success in *Chrysemys picta* eggs, and potential genetic variation in thermal tolerance. If such data could be included in this model, they could shed light on whether nest-site choice would then respond more rapidly to changing thermal conditions.

The fifth study involves simulation modelling and an analytical model to evaluate the adaptive significance of TSD in a genotypic sex determining system. The most widely accepted explanation for the evolution and persistence of TSD is based on a model by Charnov and Bull (1977), positing that some incubation environments provide higher fitness to one sex, whereas other incubation environments provide higher fitness to the other sex (Shine, 1999). This differential fitness benefit has not been widely or convincingly demonstrated in reptiles with TSD (Shine, 1999). My models demonstrated that TSD can invade a population exhibiting GSD and reach fixation through several avenues: as an extrachromosomal sex ratio distorter, as a relatively neutral mechanism with no adaptive

significance in long-lived organisms, or as a "spoiler" that interferes with the original GSD system by producing YY individuals with reduced fitness. TSD can also invade and be maintained in a GSD population through biasing sex ratios or through natal philopatry, but cannot reach fixation in these scenarios. These results demonstrate that an adaptive explanation for TSD is not necessary for TSD to evolve out of GSD.

In sum, these studies provide evidence that adaptive and facultative adjusting of sex ratios is weak, at best, in reptiles with temperature-dependent sex determination. Females do not nest in environments exhibiting extreme soil temperatures (Chapter 2), suggesting that they select intermediate sites less likely to bias sex ratios. Thermal sensitivity of offspring sex may contribute in part to the maintenance of sex ratios (Chapter 3), but differences among populations were small. Pivotal temperatures from most *C. picta* populations may reflect minor adjustments in response to differences in female nest-site choice (Ewert et al., 1994). Even if females with TSD nest in similar thermal environments across their ranges (Chapter 4), they may primarily select nest sites based mainly on other factors (e.g., hydric conditions or predation risk), reflecting selective forces other than Fisherian sex ratio selection. Investigating patterns of nest-site selection in TSD and closely related GSD taxa may shed more light on this possibility. Female *C. picta* may have little control over influencing the thermal environment that their offspring experience, but doing so through natal philopatry is likely to be maladaptive in terms of sex ratio evolution (Chapter 5). The possibility that TSD may persist as a neutral mechanism (and even invade a GSD population as such, Chapter 6) suggests that TSD may have no adaptive significance, and sex ratio adjustments through behavioral traits may not be necessary for TSD to evolve and persist in stable climatic conditions. Perhaps the most likely mechanisms allowing

reptiles with TSD to persist through rapid environmental changes are changing timing of nesting, relatively minor adjustments in pivotal temperatures, or through range expansions. Nest-site selection could certainly also respond to biased sex ratios caused by environmental changes, but likely responds more readily to other selective pressures. Given projected climate scenarios for the next century (e.g., Zwiers, 2001), we may be fortunate (or more likely unfortunate) enough to directly observe the outcome employed by reptiles with TSD.

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